STUDIES ON THE METABOLIC AND FUNCTIONAL CONSEQUENCES OF ISCHAEMIA IN THE MAMMALIAN MYOCARDIUM

Submitted in accordance with the requirements for the degree

of

Doctor of Philosophy

by

BRENDAN CLARKE

The University of Glasgow

Institute of Biochemistry

September 1993

The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others

ProQuest Number: 13833422

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

ProQuest 13833422

Published by ProQuest LLC (2019). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

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

Thesis
9677
Copy 1

For

Barbara

Jennifer and Richard

SUMMARY

The metabolic and functional effects of hypoxia, low-flow and no-flow ischaemia were studied and compared in the isolated perfused guinea-pig heart. The low-flow ischaemia model was identified as a suitable experimental system for investigating the role of glucose and fatty acids as metabolic substrates during ischaemia.

Hypoxia and ischaemia caused a reduction in high energy phosphate and glycogen in the perfused heart. These conditions also resulted in a rapid loss of contractile force and changes to diastolic tension (contracture development). Substrate availability, heart rate, and the degree of ischaemia affected the metabolic and contractile responses. The effects of impaired glucose metabolism were demonstrated by perfusing the heart in the absence of exogenous glucose.

Contracture development was characteristic of conditions in which the glycolytic capacity of the ischaemic heart was shown to be reduced (e.g. under glucose-free conditions and in no-flow ischaemia). Palmitate in the presence of glucose caused contracture developement in the low-flow ischaemic heart. Palmitate also increased the myocardial long-chain acylcarnitine content which may increase ischaemic cell damage. The inhibitory effects of etomoxir on palmitoyl carnitine transferase-I activity were demonstrated in isolated mitochondria and the perfused heart. Albumin binding prevented further investigations into the beneficial effects of reducing fatty acid oxidation by this mechanism.

ATP-dependent potassium channel activation was investigated as a possible mechanism of early contractile failure (ECF). The effects of cromakalim in the guinea-pig papillary muscle preparation were inhibited by glibenclamide. Glibenclamide did not prevent ECF during hypoxia in this preparation.

The effects of the novel anti-ischaemic agent, ranolazine (Syntex), were investigated in the low-flow ischaemia model. Ranolazine preserved the myocardial ATP content and reduced lactate and creatine kinase release during the ischaemic period. These protective effects were associated with an increased level of active pyruvate dehydrogenase.

The studies presented in this thesis have examined some of the potential mechanisms underlying the responses to hypoxia and ischaemia in the isolated perfused guinea-pig heart and papillary muscle. These results indicate that stimulation of glucose metabolism may be an important intervention in reducing ischaemic damage in the myocardium. Therapeutic strategies to achieve this are discussed.

Acknowledgements

The studies reported in this thesis were performed in the Department of Pharmacology, Syntex Research Centre, Edinburgh, in conjunction with the Institute of Biochemistry, University of Glasgow. Neither this thesis nor any part of it has been submitted to any other University.

My thanks to Syntex for their financial and practical assistance during the completion of this thesis. To Drs. Leslie Patmore and Jocelyn Dow for their sustained support and guidance throughout the duration of this work. To Dr. Jim McCormack without whose encouragement and never faltering enthusiasm, I would still be typing.

My thanks and appreciation to all those who have helped me with these studies. To Chic Calder for his photographic skills, to Stuart Fraser for introducing me to electrophysiology, to Alec Collins and Liz Shaw for the enzyme analysis and to Katrina Wyatt, whose effervescence (and LCA assay) repeatedly revived my failing spirit.

Finally, to my family, who have endured the effort of this task with love and encouragement and who now, like me, will rest in peace.

Contents

Title page

Dedication

Summary

Acknowledgements

Contents

List of figures and tables

Abbreviations

	Page
Chapter One	1
General introduction	
Introduction	2
1.1 Myocardial ischaemia	3
1.1.1 Angina	3
1.1.2 Aetiology of angina	3
1.1.3 The clinical syndromes of angina	4
1.2 Metabolism in the normoxic myocardium	5
1.2.1 General considerations	5
1.2.2 The control of glycolysis	6
1.2.3 Lipid metabolism	9
1.2.4 The tricarboxylic acid (TCA) cycle and oxidative phosphorylation	14
1.3 The effects of ischaemia on myocardial function and metabolism	16
1.3.1 Cell structure	16

1.3.2 Contractile function 17

1.3.2.1 Factors contributing to loss of contractile force 17

1.3.2.2 Factors contributing to contracture development 20

1.3.3 Metabolic function in ischaemia 23

1.4 Clinical treatment of myocardial ischaemia 34

1.5 Aims of this thesis 35

Chapter Two 37

Materials and Methods

2.1 Biochemicals and chemicals 38

2.2 Experimental animals 38

2.3 Methods 38

2.3.1 The Langendorff-perfused guinea-pig heart preparation 38

2.3.2 The isolated guinea-pig papillary muscle preparation 40

2.3.3 Tissue metabolite extraction 43

2.3.4 Biochemical determination of enzyme activity and metabolite concentration 44

2.3.5 Conversion of wet to dry tissue weight 52

2.3.6 Preparation of palmitate solution 53

2.3.7 Electrophysiological measurements in the papillary muscle preparation 53

2.3.8 Preparation of heart mitochondria 54

Chapter Three 55

Characterisation of ischaemic and hypoxic models in the guinea-pig perfused heart preparation

3.1 Introduction 56

3.2 Results and discussion 57

3.2.1 The perfused guinea-pig heart preparation 57

3.2.1.1 Contractile function and the metabolic characteristics of the perfused heart at the end of the equilibration period 57

3.2.2 The effects of hypoxia 58

3.2.2.1 The effects of hypoxia on the contractile function of the unpaced perfused heart 58

3.2.2.2 The effects of hypoxia on the metabolic characteristics of the unpaced perfused heart 63

3.2.2.3 The effects of hypoxia on the contractile function of the paced perfused heart 64

3.2.2.4 The effects of hypoxia on the metabolic characteristics of the paced perfused heart 64

3.2.3 The effects of no-flow ischaemia

3.2.3.1 The effects of no-flow ischaemia on the contractile function of the unpaced perfused heart 67

3.2.3.2 The effects of no-flow ischaemia on the metabolic characteristics of the unpaced perfused heart 68

3.2.3.3 The effects of no-flow ischaemia on the contractile function of the paced perfused heart 69

3.2.3.4 The effects of no-flow ischaemia on the metabolic characteristics of the paced perfused heart 69

3.2.4 The effects of low-flow ischaemia

3.2.4.1 The effects of low-flow ischaemia on the contractile function of the unpaced perfused heart 72

3.2.4.2 The effects of low-flow ischaemia on the metabolic characteristics of the unpaced perfused heart 73

3.2.4.3 The effects of low-flow ischaemia on the contractile function of the paced perfused heart 74

3.2.4.4 The effects of low-flow ischaemia on the metabolic characteristics of the paced perfused heart 74

3.3 Discussion 79

Chapter Four 83

Studies on glucose and fatty acid metabolism during normoxia, hypoxia and ischaemia in the guinea-pig perfused heart preparation

4.1 Introduction 84

4.2 Results and discussion 85

4.2.1 The effects of glucose-free perfusion in the normoxic-perfused heart 85

4.2.1.1 The effects of glucose-free perfusion on the contractile function of the normoxic-perfused heart 85

4.2.1.2 The effects of glucose-free perfusion on the metabolic characteristics of the normoxic-perfused heart 90

4.2.2 Studies on the mechanism of fibrillation observed in the normoxic-perfused heart during glucose-free perfusion 91

4.2.2.1 The high-energy phosphate and glycogen content during fibrillation in the normoxic heart perfused in the absence of glucose 91

4.2.2.2 Measurement of the cardiac action potential duration in the guinea-pig papillary muscle preparation during glucose-free superfusion 94

4.2.3 The effects of glucose-free perfusion in the paced hypoxic and low-flow ischaemic perfused heart 97

4.2.3.1 The effects of glucose-free perfusion on the contractile function of the paced hypoxic-perfused heart 97

4.2.3.2 The effects of glucose-free perfusion on the metabolic characteristics of the paced hypoxic-perfused heart 98

4.2.3.3 The effects of glucose-free perfusion on the contractile function of the paced low-flow ischaemic-perfused heart 98

4.2.3.4 The effects of glucose-free perfusion on the metabolic characteristics of the paced low-flow ischaemic-perfused heart 103

4.2.4 Lactate production in the paced ischaemic heart 103

4.2.4.1 Low-flow ischaemia in the presence of glucose 103

4.2.4.2 Low-flow ischaemia in the absence of glucose 106

4.2.4.3 No-flow ischaemia 106

4.2.5 Contracture development in the paced low-flow ischaemic heart perfused in the absence of glucose 109

4.2.5.1 The metabolic characteristics of the glucose-free perfused low-flow ischaemic heart at the onset of contracture 110

4.2.5.2 The potential consequences of contracture development in the paced perfused heart during low-flow ischaemia 110

4.3 Lipid metabolism in the normoxic and ischaemic perfused heart 116

4.3.1 The LCA content of the paced normoxic and low-flow ischaemic perfused heart 117

4.3.1.1 The effects of albumin and albumin-bound palmitate on contractile function in the paced perfused heart during low-flow ischaemia 120

4.3.1.2 The metabolic characteristics of the paced heart following perfusion with albumin and albumin-bound palmitate 123

4.3.2 The effects of albumin and albumin-bound palmitate during low-flow ischaemia in the paced heart perfused with 5mM glucose 123

4.3.2.1 The effects of albumin and albumin-bound palmitate on contractile function during low-flow ischaemia in the paced heart perfused with 5mM glucose 124

4.3.2.2 The effects of albumin and albumin-bound palmitate on the metabolic characteristics of the paced low-flow ischaemic heart perfused with 5mM glucose 127

4.3.3 Inhibition of myocardial CPT-1 by etomoxir in the isolated mitochondria preparation 133

4.3.4 Evidence for CPT-I inhibition by etomoxir in the paced normoxic-perfused heart 133

4.3.5 The potential effects of albumin-binding in the perfused heart studies 134

4.3.5.1 The effects of albumin on the positive inotropic response to palmitoylcarnitine in the guinea-pig papillary muscle preparation 137

4.3.6 The effects of etomoxir in the normoxic-perfused heart 140

4.3.6.1 The effects of etomoxir on contractile function in the unpaced normoxic heart perfused with and without glucose 140

4.3.6.2 The effects of etomoxir on the glycogen content of the unpaced normoxic heart perfused with and without glucose 143

4.4 Discussion 144

Chapter Five 147

The effects of ranolazine, a novel anti-ischaemic agent, in the normoxic and ischaemic myocardium

5.1 Introduction 148

5.2 Methods 149

5.2.1 The guinea-pig papillary muscle preparation 149

5.2.1.1 Ranolazine vs elevated calcium concentration 149

5.2.1.2 Ranolazine vs Bay K 8644 149

5.2.1.3 Ranolazine vs orciprenaline 149

5.2.1.4 Ranolazine vs forskolin 150

5.2.2 The perfused guinea-pig heart preparation 151

5.3 Results and discussion 152

5.3.1 The effects of ranolazine in the guinea-pig papillary muscle preparation 152

5.3.1.1 The effects of ranolazine on contractile force prior to the addition of elevated $[Ca^{++}]_o$ or Bay K 8644 152

5.3.1.2 The effects of ranolazine on the positive inotropic response to elevated $[Ca^{++}]_o$ or Bay K 8644 155

5.3.1.3 The effects of ranolazine on contractile force prior to the addition of orciprenaline 155

5.3.1.4 The effects of ranolazine on the positive inotropic response to orciprenaline 158

5.3.1.5 The effects of ranolazine on the positive inotropic response to forskolin 161

5.3.1.6 The effects of ranolazine on cyclic AMP concentrations and the positive inotropic response to forskolin 164

5.3.2 The effects of ranolazine on the paced low-flow ischaemic perfused guinea-pig heart 167

5.3.2.1 The effects of ranolazine on contractile function during pre-treatment and low-flow ischaemia 167

5.3.2.2 The effects of ranolazine on the metabolic characteristics of the low-flow ischaemic heart 172

5.4 Discussion 177

<u>Chapter Six</u> 179

<u>The involvement of ATP-dependent potassium channel activation in early contractile failure during cardiac ischaemia and hypoxia *in vitro*</u>

6.1 Introduction 180

6.2 Methods 185

6.2.1 The guinea-pig perfused heart preparation

6.2.2 The guinea-pig papillary muscle preparation 185

6.2.2.1 Measurement of the effective refractory period 185

6.2.2.2 Experimental conditions in hypoxic studies using the paced guinea-pig papillary muscle preparation 188

6.3 Results and discussion 191

6.3.1 The ECF response during ischaemia and hypoxia in the paced perfused guinea-pig heart 191

6.3.2 The effect of glibenclamide in the paced perfused guinea-pig heart 196

6.3.3 The effect of cromakalim in the paced guinea-pig papillary muscle preparation 196

6.3.4 Inhibition of the effects of cromakalim by glibenclamide in the paced guinea-pig papillary muscle preparation 201

6.3.5 The effects of glibenclamide on the response to hypoxia in the paced guinea-pig papillary muscle preparation 206

6.3.6 The effects of glibenclamide on the response to prolonged hypoxia in the paced guinea-pig papillary muscle preparation 211

6.4 Discussion 214

Chapter Seven 217

Epilogue: The potential of metabolic intervention for future drug development

7.1 The role of metabolic substrates in cardiac ischaemia 218

7.2 Strategies to alter cardiac metabolism during ischaemia 220

7.2.1 Targetting fatty acid metabolism 220

7.2.2 Targetting glucose metabolism 222

Bibliography 225

Publications 241

List of Figures and Tables

Chapter One

		Page
Figure 1.1	The glycolytic pathway	7
Figure 1.2	Triglyceride synthesis	12
Figure 1.3	ß-oxidation of fatty acids	13
Figure 1.4	The tricarboxylic acid cycle	15
Figure 1.5	The cardiac action potential configuration	19
Figure 1.6	The enzymatic control of glycogen metabolism	27

Chapter Two

Figure 2.1	The Langendorff heart and papillary muscle perfusion apparatus	42

Chapter Three

Figure 3.1	The effects of hypoxia on the contractile function of unpaced and paced perfused guinea-pig hearts	60
Figure 3.2	The comparative effects of hypoxia, no-flow and low-flow ischaemia on the spontaneous beating rate in unpaced perfused guinea-pig hearts	62
Figure 3.3	The effects of no-flow ischaemia on the contractile function of unpaced and paced perfused guinea-pig hearts	66
Figure 3.4	The effects of low-flow ischaemia on the contractile function of unpaced and paced perfused guinea-pig hearts	71
Table 3.1	The effects of hypoxia, no-flow ischaemia and low-flow ischaemia on the metabolic characteristics of unpaced perfused hearts	76

Table 3.2 The effects of hypoxia, no-flow ischaemia and low-flow ischaemia on the metabolic characteristics of paced perfused hearts 78

Chapter Four

Figure 4.1 The effects of glucose-free perfusion on contractile force and diastolic tension in the paced normoxic perfused guinea-pig heart 87

Figure 4.2 The metabolic status of the paced guinea-pig heart after glucose-free normoxic perfusion 89

Figure 4.3 The effects of glucose-free superfusion on the action potential duration in the normoxic guinea-pig papillary muscle 93

Figure 4.4 The effects of glucose-free hypoxia on contractile force and diastolic tension in the paced perfused guinea-pig heart 96

Figure 4.5 Comparison of the effects of low-flow ischaemia with and without glucose on contractile function in the paced perfused guinea-pig heart 100

Figure 4.6 Time-dependent lactate efflux during low-flow ischaemia with and without glucose in the paced perfused guinea-pig heart 105

Figure 4.7 Comparison of tissue lactate accumulation during low-flow and no-flow ischaemia in the paced perfused guinea-pig heart 108

Figure 4.8 The effect of glucose on contracture development during low-flow ischaemia in the paced perfused guinea-pig heart 112

Figure 4.9	The effect of contracture development on myocardial perfusion during low-flow ischaemia with and without glucose in the paced perfused guinea-pig heart	115
Figure 4.10	Comparison of the contractile changes in the paced normoxic and low-flow ischaemic guinea-pig heart in the presence of albumin or albumin-bound palmitate	119
Figure 4.11	Long-chain acylcarnitine content and creatine kinase release in the paced normoxic, low-flow ischaemic and low-flow ischaemic guinea-pig heart perfused with albumin or albumin-bound palmitate	122
Figure 4.12	Comparison of the contractile changes in the paced low-flow ischaemic guinea-pig heart perfused with albumin or albumin-bound palmitate in the presence of 5mM glucose	126
Figure 4.13	Lactate and creatine kinase release in the paced low-flow ischaemic guinea-pig heart perfused with albumin or albumin-bound palmitate in the presence of 5mM glucose	129
Figure 4.14	The effect of etomoxir on palmitoylcarnitine transferase 1 (CPT-1) activity in rat and guinea-pig heart mitochondria	132
Figure 4.15	The effect of albumin on the positive inotropic activity of palmitoyl carnitine in the paced superfused guinea-pig papillary muscle preparation	136
Figure 4.16	The effects of etomoxir on contractile activity in the paced guinea-pig heart during normoxic perfusion with and without glucose	139

Figure 4.17	The effects of etomoxir on glycogen levels in the paced guinea-pig heart during normoxic perfusion with and without glucose	142
Table 4.1	The comparative effects of hypoxia and low-flow ischaemia on the metabolic status of paced guinea-pig hearts perfused in the absence of glucose	102

Chapter Five

Figure 5.1	The effect of ranolazine on the positive inotropic response to Bay K 8644 in the paced guinea-pig papillary muscle preparation	154
Figure 5.2	The comparative effects of ranolazine, nicardipine and nifedipine on contractile activity in the paced guinea-pig papillary muscle preparation	157
Figure 5.3	The effect of ranolazine on the positive inotropic response to orciprenaline in the paced guinea-pig papillary muscle preparation	160
Figure 5.4	The effects of ranolazine on the positive inotropic response to forskolin in the paced guinea-pig papillary muscle preparation	163
Figure 5.5	The effects of ranolazine on cyclic AMP and the positive inotropic response to forskolin in the paced guinea-pig papillary muscle preparation	166
Figure 5.6	The effects of ranolazine on contractile function during pre-treatment and low-flow ischaemia in the paced perfused guinea-pig heart	169
Figure 5.7	The effects of ranolazine on lactate and creatine kinase release during low-flow ischaemia in the paced perfused guinea-pig heart	174

Figure 5.8	The comparative effects of ranolazine on ATP and PDHa following low-flow ischaemia in the paced perfused guinea-pig heart	176
Table 5.1	The effects of ranolazine on the metabolic characteristics of paced low-flow ischaemic perfused guinea-pig hearts	171

Chapter Six

Figure 6.1	The cardiac action potential and related transmembranal ionic changes	182
Figure 6.2	Effective refractory period (ERP) measurements in the paced guinea-pig papillary muscle preparation	184
Figure 6.3	The comparative effects of repeated hypoxia in the paced guinea-pig papillary muscle preparation	187
Figure 6.4	Early contractile failure (ECF) in response to ischaemia and hypoxia in the paced perfused guinea-pig heart	190
Figure 6.5	The effect of glibenclamide on cardiac function during normoxic perfusion in the paced perfused guinea-pig heart	193
Figure 6.6	Time-dependent changes of contractile force and ERP in the paced guinea-pig papillary muscle preparation superfused under normoxic conditions	195
Figure 6.7	The effects of cromakalim on contractile force and ERP in the paced guinea-pig papillary muscle preparation superfused under normoxic conditions	198
Figure 6.8	Time-dependent effects of cromakalim on contractile force and ERP in the paced guinea-pig papillary muscle preparation superfused under normoxic conditions	200

Figure 6.9 The effect of glibenclamide on the response to cromakalim in the paced guinea-pig papillary muscle preparation superfused under normoxic conditions 203

Figure 6.10 The effects of cromakalim on contractility and ERP following pre-treatment with glibenclamide in the paced guinea-pig papillary muscle preparation superfused under normoxic conditions 205

Figure 6.11 The effects of glibenclamide on the response to hypoxia in the paced guinea-pig papillary muscle preparation 208

Figure 6.12 The effects of glibenclamide on ATP depletion during hypoxia in the paced guinea-pig papillary muscle preparation 210

Figure 6.13 The effects of glibenclamide on the response to prolonged hypoxia in the paced guinea-pig papillary muscle preparation 213

Chapter Seven

Table 7.1 Summary of the effects of substrate utilisation in the ischaemic myocardium 224

Abbreviations

The abbreviations used in this thesis are in accordance with the guidelines set down in the Biochemical Journal Instructions to Authors unless defined below

H^+, Ca^{++}, Na^+, Mg^{++}, Cl^-, K^+ refer to the free ionic species of hydrogen, calcium, sodium, magnesium, chloride and potassium, respectively

AABS	p - (p-aminophenylazobenzene sulphonic acid)
APD	action potential duration
AUC	area under the curve
cAMP	cyclic AMP
CK	creatine kinase
CPT-I	carnitine palmitoyl transferase-I
CrP	creatine phosphate
DCA	dichloroacetate
diP	di-phosphate
ECF	early contractile failure
ERP	effective refractory period
$[\ ]_o$	extracellular
Glib	glibenclamide
IU	international unit
$[\ ]_i$	intracellular
K_{ATP}	ATP-dependent potassium channel
Ki	$IC_{50}/\ (1+([L]/Kd)$
LCA	long chain acylcarnitine
LDH	lactate dehydrogenase
LFI	low-flow ischaemia
mosmol/Kg	mmols per Kg water
msec	millisecond
NEFA	non-esterified (free) fatty acids
NFI	no-flow ischaemia
PC	palmitoyl carnitine
PDH	pyruvate dehydrogenase complex
PDHa	dephosphorylated, active form of PDH
PDHt	total PDH activity
pIC_{50}	-log molar concentration required to reduce response by 50%
pKi	-log Ki
P/O	phosphorylation/oxidation ratio
PO_2	partial pressure of oxygen
PSS	physiological salt solution
PTFE	polytetrafluorethylene
S.E.	standard error of estimate of mean value
SR	sarcoplasmic reticulum
TCA	tricarboxylic acid cycle

CHAPTER ONE

GENERAL INTRODUCTION

INTRODUCTION

Cardiovascular disease remains one of the major causes of mortality in man and the past decade has seen a dramatic increase in the development of new chemical entities for the management of cardiovascular disease. At the present time, three main groups of cardioprotective drugs exist-

1) Drugs for treatment of heart failure (e.g.digoxin)
2) Antiarrhythmic drugs (e.g. phenytoin, amiodarone)
3) Drugs for the treatment of cardiac ischaemia

Drugs used predominantly in the treatment of cardiac ischaemia, which is the topic of this thesis, include ß-adrenoceptor blocking agents, e.g. propranolol, atenolol, which were first developed in the late 1950's (Moran and Perkins, 1958), vasodilating agents e.g. nitrates (Davis, 1955) and more recently calcium antagonists (Fleckenstein, 1977).

While improving cellular function during ischaemia, the action of these drugs is indirect in that their main effect is to improve the efficiency of myocardial oxygen utilisation either through increased blood flow or a reduction in cardiac workload. Much research, including that detailed in this thesis, is directed at investigations into the possible benefits of drug intervention. Through a direct action at the level of cell metabolism it may be possible to improve the specificity of drug action and reduce the side-effects associated with existing therapies.

This chapter presents a review of the effects of ischaemia on myocardial function and metabolism in order to evaluate the potential of this novel approach to the treatment of myocardial ischaemia.

1.1 MYOCARDIAL ISCHAEMIA

1.1.1 Angina

Cardiac ischaemia represents an imbalance between the myocardial demand for oxygen and the vascular supply of coronary blood. This condition, which exists when the uptake of oxygen in the heart is insufficient to maintain the normal rate of cellular oxidation, was first described by William Heberden in 1772 (Heberden, 1772). His observations defined the classical symptoms of the ischaemic syndrome which is now known as *angina pectoris*. Since these early observations, three main types of angina have been classified (see Section 1.1.3):

1) Variant (Prinzmetal) angina.
2) Effort (chronic stable) angina.
3) Unstable angina.

1.1.2 Aetiology of angina

Angina has been defined as " a sense of discomfort arising in the myocardium as a result of myocardial ischaemia in the absence of infarction " (Julian, 1982). This syndrome can arise from vascular disorders including, i) vasospasm which results in a sudden reduction in coronary artery diameter and subsequent perfusion (d'Hemecourt and Detour, 1978), ii) vasoconstriction due to increased vascular sensitivity to physiological stimuli (Kawachi *et al.*, 1984) resulting in restriction of coronary blood flow to vital areas of the myocardium or iii) an increased oxygen requirement of the heart which exceeds the existing maximum perfusion capacity of the coronary circulation (Marzilli *et al.*, 1978). This may arise from a reduced perfusion capacity due to the presence of artherosclerotic lesions or as a consequence of platelet aggregation (Davies, 1990).

1.1.3 The clinical syndromes of angina

Variant (Prinzmetal) Angina

In 1959, Prinzmetal described an anginal condition which occurred at rest and was accompanied by elevation of the ST-segment of the electrocardiogram (Prinzmetal *et al.*, 1959). Subsequent investigations identified the most frequent cause as vasospasm (Meller *et al.*, 1976; Maseri *et al.*, 1978). This was mediated through existing or developing atherosclerosis (Kawachi *et al.*, 1984) or other endogenous agents which restricted flow through the coronary vessels (Sellke *et al.*, 1990; Yang *et al.*, 1990).

Effort (Chronic stable) Angina

Effort angina differs from Prinzmetal's angina in that coronary perfusion is limited by a permanent restriction usually due to artherosclerotic lesions. The effects of reduced flow may not be apparent until the oxygen requirements of the heart are raised by exercise or some other stimuli (Nayler, 1988).

Unstable Angina

Unstable angina is characterised by a sudden increase in the severity of an existing anginal condition (Fowler, 1971) and is a condition associated with obstructive coronary artery disease, increased vasoactivity and thrombosis (Packer, 1989). Clinically, the severity of this condition lies between stable effort angina and acute myocardial infarction and early observations have recognised that myocardial infarction is frequently preceded by angina which has developed or progressed rapidly (Herrick, 1912; Kahn, 1926).

Myocardial ischaemia is common to all anginal syndromes, irrespective of their aetiology and the underlying mechanisms involve plaque formation (Garlin *et al*, 1986; Feldman, 1987) although coronary artery spasm can occur in patients with normal or near normal arteries.

Myocardial Infarct

The biochemical consequences of ischaemia are multiple and arise from a

complex of interrelated metabolic changes which have been extensively reviewed (Hearse, 1980; Hillis and Braunwald, 1977). The effects of ischaemia are dependent on both the duration and degree of the ischaemic insult (Jennings *et al.*, 1990). It is generally accepted that, under experimental conditions, short durations of ischaemia (15 mins) produce changes to myocardial cell function which can be reversed on reperfusion. Longer periods of ischaemia (40-60 mins) induce changes which are not reversed on reperfusion. In fact, reperfusion at this stage can result in increased myocardial cell damage and necrosis leading to the formation of an area of myocardial infarction (Otani *et al.*, 1984).

Clinically, the major cause of ischaemia is blockade of major coronary blood vessels. However, the degree of infarction is usually smaller than the area perfused by the occluded artery because of influential factors such as vasodilatation and the existence and initiation of collateral flow (Rivas *et al.*, 1976; Jugdutt *et al.*, 1979).

1.2 METABOLISM IN THE NORMOXIC MYOCARDIUM

1.2.1 General considerations

One aspect of this thesis is to examine the metabolic consequences of cardiac ischaemia on cardiac metabolism. In order to do this, it is first necessary to review the normal metabolic processes of the heart.

ATP is used in the activity of various intracellular enzymes, to maintain ionic equilibrium in the cell and to sustain the contractile process of the myocardium. The major substrates for the production of ATP in myocardial cells are glucose and fatty acids although lactate, ketone bodies and proteins are also utilised. Limited amounts of endogenously stored glycogen and triglyceride are also available. ATP can be generated in the myocardium by the main metabolic pathways of glycolysis (anaerobic) and oxidative phosphorylation (aerobic). In the normal myocardium, the production of ATP is strictly coupled to myocardial oxygen consumption

(LaNoue and Schoolworth, 1979) and the majority of myocardial ATP (90%) is produced by oxidative phosphorylation (Kobayashi and Neely, 1979). Before glucose can enter the TCA cycle, it must pass through the glycolytic pathway where some ATP can be produced even in the absence of oxygen. Under aerobic conditions, the metabolism of 1 mole of glucose, via glycolysis and the tricarboxylic acid (TCA) cycle, generates 38 moles of ATP. The production of ATP from fatty acid oxidation is dependent on the carbon chain length and, on a molar basis, is much greater than that from glucose oxidation. For example, the complete oxidation of 1 mole of palmitate can produce 130 moles of ATP. However, the P/O ratio for the oxidation of lipid (2.83) is lower than that of glucose (3.17) indicating that more oxygen (~12%) is required to completely oxidise fat compared to carbohydrate (Opie, 1991). Lipids undergo ß-oxidation prior to oxidation in the tricarboxylic acid (TCA) cycle and can only be metabolised to ATP under aerobic conditions.

1.2.2 The control of glycolysis

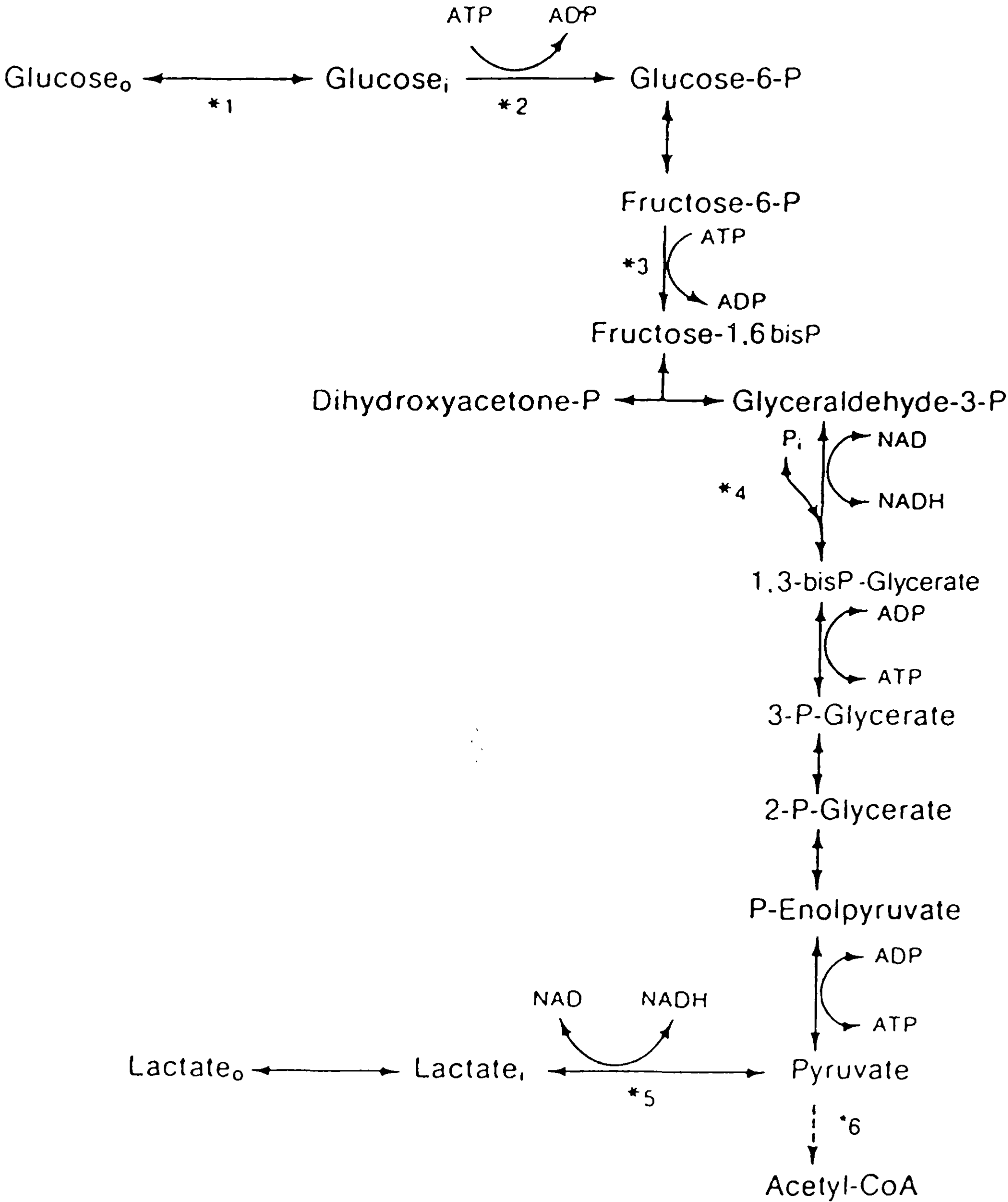
The entry of glucose into the myocardial cell is facilitated by a carrier-mediated system which can be stimulated by external factors such as insulin and adrenaline. The metabolism of 1 molecule of glucose in the glycolytic pathway yields a net synthesis of 2 molecules of ATP per 6-carbon sugar and produces acetyl-CoA under normoxic conditions or lactate under anaerobic conditions (Figure 1.1).

The major control points affecting the glycolytic rate are: 1) glucose transport, 2) hexokinase, 3) phosphofructokinase, 4) glyceraldehyde-3-phosphate dehydrogenase, 5) lactate dehydrogenase , 6) pyruvate dehydrogenase .

1) Glucose transport

The rate of uptake of glucose can be modified to meet the energy requirements of the cell (e.g. insulin) and is also influenced by alternative substrate availability (Randle *et al.*, 1963).

Figure 1.1 The Glycolytic Pathway



2) Hexokinase

This reaction catalyses glucose phosphorylation and uses 1 molecule of ATP in the process. The activity of hexokinase is inhibited by high concentrations of glucose-6-phosphate which allosterically modifies the enzyme. High ATP levels inhibit hexokinase activity, low ATP and high ADP, AMP and P_i stimulate activity, consequently glucose phosphorylation varies with the energy status of the cell.

3) Phosphofructokinase

Possibly the key enzyme in the control of glycolytic rate, phosphofructokinase catalyses the formation of fructose-1,6, bisphosphate from fructose-6-phosphate. This reaction also utilises one molecule of ATP and phosphofructokinase activity can be affected by a number of factors. Citrate, an intermediate of the TCA cycle has an important inhibitory effect on phosphofructokinase which slows glycolysis when the level of TCA cycle intermediates is high. This represents an essential factor in the control of glycolysis with respect to oxidative metabolism. Phosphofructokinase is also inhibited by decreased intracellular pH which has a critical inhibitory effect on the glycolytic rate during ischaemia (Rovetto *et al.*, 1975)

4) Glyceraldehyde-3-phosphate dehydrogenase

This enzyme catalyses the formation of 1,3, bisphosphoglycerate from glyceraldehyde-3-phosphate and NAD^+ is reduced to NADH + H^+ in the reaction. Under aerobic conditions, this reaction has no regulatory role. However, under anaerobic conditions, control of glycolysis shifts from the energy requirements of the cell (phosphofructokinase) to the ability of the cell to oxidise substrates. When the heart becomes unable to oxidise NADH, accumulation of NADH slows the glycolytic rate at this step in the pathway.

5) Lactate dehydrogenase

Under aerobic conditions, pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase. In the aerobic heart, lactate produced by muscle activity is extracted from the blood and subsequently oxidised. Under anaerobic conditions,

if NADH accumulates, the reduction of pyruvate to lactate by lactate dehydrogenase allows a limited capacity to regenerate NAD^+ from NADH and further glycolysis is achieved. The production of lactate by this reaction coupled with the reduction of lactate oxidation during ischaemia leads to high lactate levels in the bloodstream.

6) Pyruvate dehydrogenase (PDH)

Pyruvate crosses the inner mitochondrial membrane and is oxidised to acetyl-CoA by PDH. PDH is a multienzyme complex containing 3 component enzymes, a decarboxylase, a dihydrolipoamide acetyltransferase and a dihydrolipoamide dehydrogenase. Together, this complex catalyses the formation of acetyl CoA, NADH and CO_2 from pyruvate. PDH phosphorylation and dephosphorylation together with product inhibition regulate PDH activity in the heart. Phosphorylation of PDH, catalysed by PDH kinase, inactivates the enzyme. NADH also promotes phosphorylation of PDH and consequently inhibits PDH kinase activity indirectly. This effect of NADH can be reversed by NAD^+. PDH phosphate phosphatase reactivates PDH . During ischaemia, when NADH and acetyl-CoA accumulate, the formation of more acetyl CoA is inhibited and lactate is preferentially produced. The phosphatase is also inhibited by citrate. This inhibition promotes fatty acid metabolism and represents an important regulatory mechanism for the integration of carbohydrate and lipid metabolism.

1.2.3 Lipid Metabolism

In the normoxic heart, free fatty acids account for < 0.1% of the total cellular fatty acid content (van der Vusse *et al.*, 1982). Long chain fatty acids are esterified as phospholipids (86%) or neutral lipids, primarily triglycerides (13%) (Corr *et al.*, 1984). Fatty acids are major substrates for energy metabolism in the normoxic heart (Bing *et al.*, 1954; Carlsten *et al.*, 1961) and may be used in preference to glucose (Randle *et al.*, 1963). Fatty acids increase myocardial oxygen consumption (Challoner and Steinberg, 1966; Mjos, 1971; Kjekshus and Mjos, 1972), an effect

which can be stimulated by catecholamines (Challoner and Steinberg, 1966) and is sensitive to inhibition by anti-lipolytic agents (Mjos, 1971; Mjos *et al.*, 1974; Simonsen and Kjekshus, 1978).

Fatty acid uptake

Fatty acids are transported in the plasma to the heart either as non-esterified fatty acids bound to albumin (Morrisett *et al.*, 1975) or as triacylglycerol complexed to hydrophilic lipoproteins (Fredrickson *et al.*, 1958).

The myocardium has a high capacity for the extraction of circulating NEFA. In dogs, 55% of plasma NEFA can be extracted during a single passage through the cardiac vessels (van der Vusse *et al.*, 1983) indicating the existence of an efficient uptake process. It has been suggested that the plasma NEFA:albumin ratio may be an important factor in the control of NEFA uptake and that a high NEFA:albumin ratio may result in excess unbound NEFA being transferred into heart muscle (Evans, 1964). Although a mass action effect has been proposed as the major factor controlling NEFA uptake (Spector, 1971), a more specific interaction of the NEFA-albumin complex with sites on the luminal membranes of endothelial cells in the coronary vessels has also been proposed (Little *et al.*, 1986). Rauch *et al*, (1987), have suggested that the NEFA-albumin complex binds to a sarcolemmal albumin receptor before translocation of the NEFA across the sarcolemmal membrane and specific albumin receptors have been located on capillary endothelium cells in the heart (Ghitescu *et al.*, 1986). A role for fatty acid binding protein (FABP) in the intracellular transport of NEFA has been proposed and the presence of FABP in myocardial cell types has been confirmed (Fournier and Rahim, 1985). Stremmel *et al*, (1985) have isolated a protein in cardiac cells which may facilitate NEFA transport across the capillary endothelium It has been suggested that transport of NEFA from the plasma to the site of ß-oxidation may involve a sequence of specific binding proteins. However, the precise mechanism underlying fatty acid uptake remains unclear.

Triglycerides

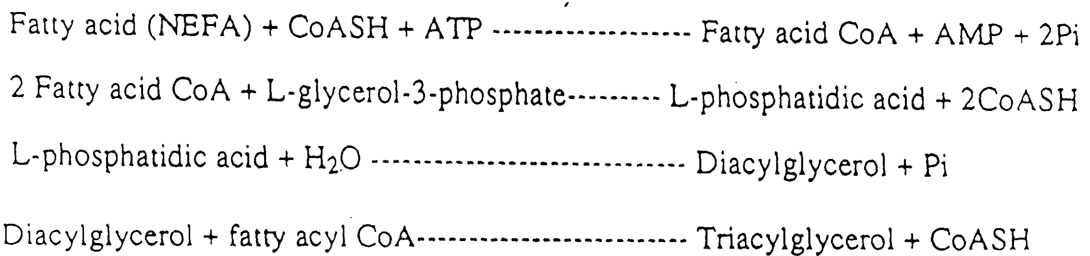
Endogenous triglycerides are a major source of substrate in the normoxic myocardium and are preferentially oxidised in the presence of glycogen (Olson and Hoeschen, 1967). These triglycerides are synthesised from glycerol-3-phosphate and fatty acyl CoA (Kennedy, 1961) and the synthesis pathway is shown in Figure 1.2.

Labelled palmitate injected into the coronary arteries of dog hearts, *in vivo*, was rapidly incorporated into the endogenous triglyceride store (Klein *et al.*, 1979) indicating that some exogenous NEFA undergoes esterification before oxidation. Synthesis and degradation of triglycerides is a cyclical process which releases glycerol and fatty acids (Shipp *et al.*, 1964). The fatty acids are subsequently activated by combination with CoASH and enter the mitochondrial ß-oxidation pathway (Groot *et al.*, 1976). The breakdown of triglycerides by hormone-sensitive lipase activity is regulated by feedback control and is subject to product inhibition (Severson and Hurley, 1982; Stam and Hulsmann, 1985).

Metabolic fate of fatty acids

After transport across the sarcolemma, NEFA molecules are either oxidised within the mitochondria or incorporated into esterified fatty acids and stored as triglycerides, phosphoglycerides or cholesteryl esters (van der Vusse *et al.*, 1987; Crass, 1972). Fatty acids are "activated" to long chain acyl-CoA esters by ATP and acyl-CoA synthetase. Long chain acylCoA is converted to long chain acylcarnitine and is subsequently translocated across the inner mitochondrial membrane (Haddock *et al* ., 1970, Brosnan and Fitz, 1971). Transfer within the mitochondria involves the activity of carnitine acyltransferase I which is located on the inner face of the outer mitochondrial membrane, the acylcarnitine: carnitine transporter and carnitine acyltransferase II which re-forms acyl-CoA prior to ß-oxidation (Pande, 1975; Ramsay and Tubbs, 1975). Carnitine acyltransferase I has been identified as a key regulating enzyme in lipid metabolism (Pauly et al., 1991) and the activity of this enzyme can be inhibited by endogenous malonyl CoA (Haddock *et al.*, 1970;

Figure 1.2 Triglyceride Synthesis

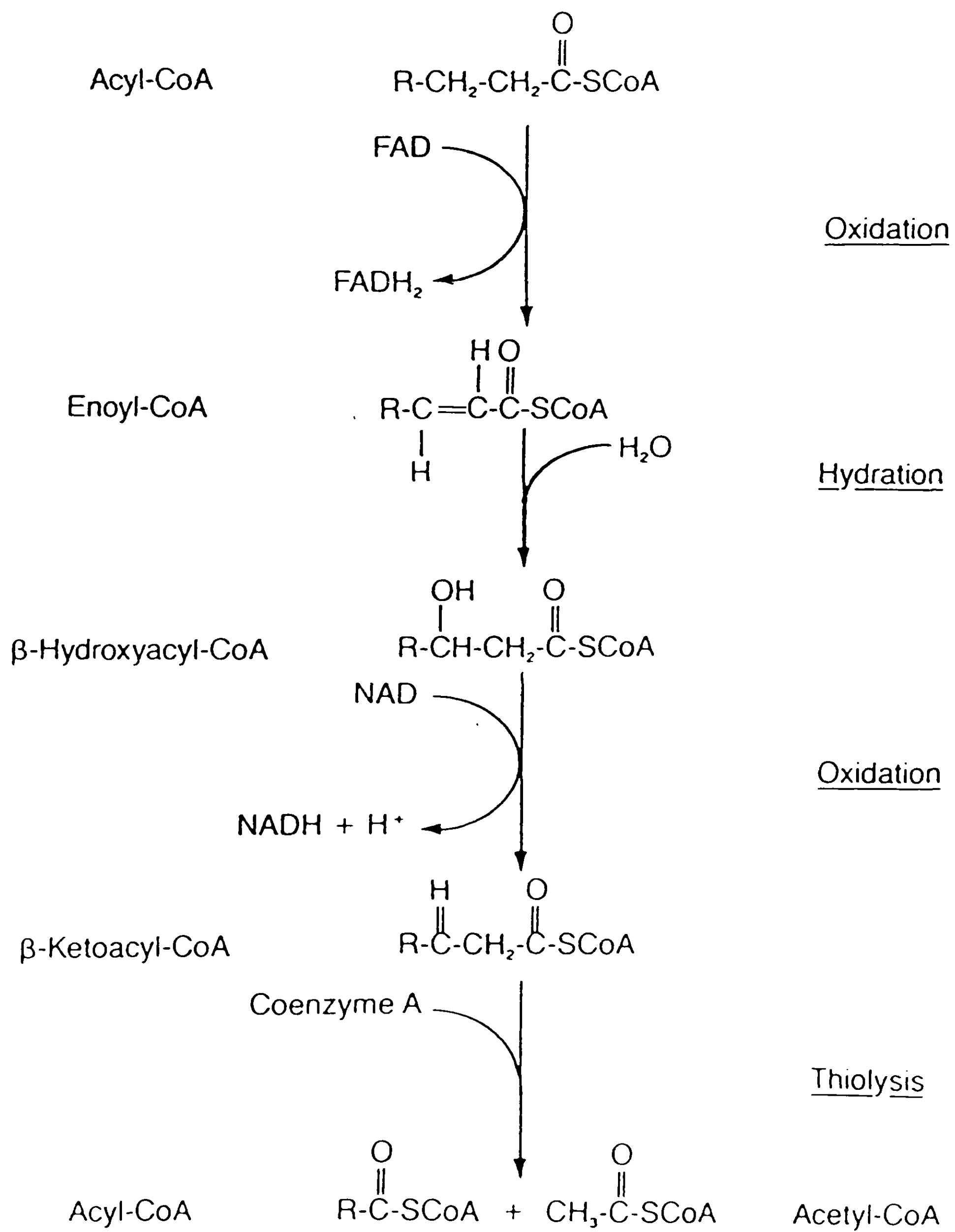

Fatty acid (NEFA) + CoASH + ATP ------------------ Fatty acid CoA + AMP + 2Pi

2 Fatty acid CoA + L-glycerol-3-phosphate--------- L-phosphatidic acid + 2CoASH

L-phosphatidic acid + H_2O ----------------------------- Diacylglycerol + Pi

Diacylglycerol + fatty acyl CoA------------------------- Triacylglycerol + CoASH

Figure 1.3 ß-oxidation of Fatty Acids



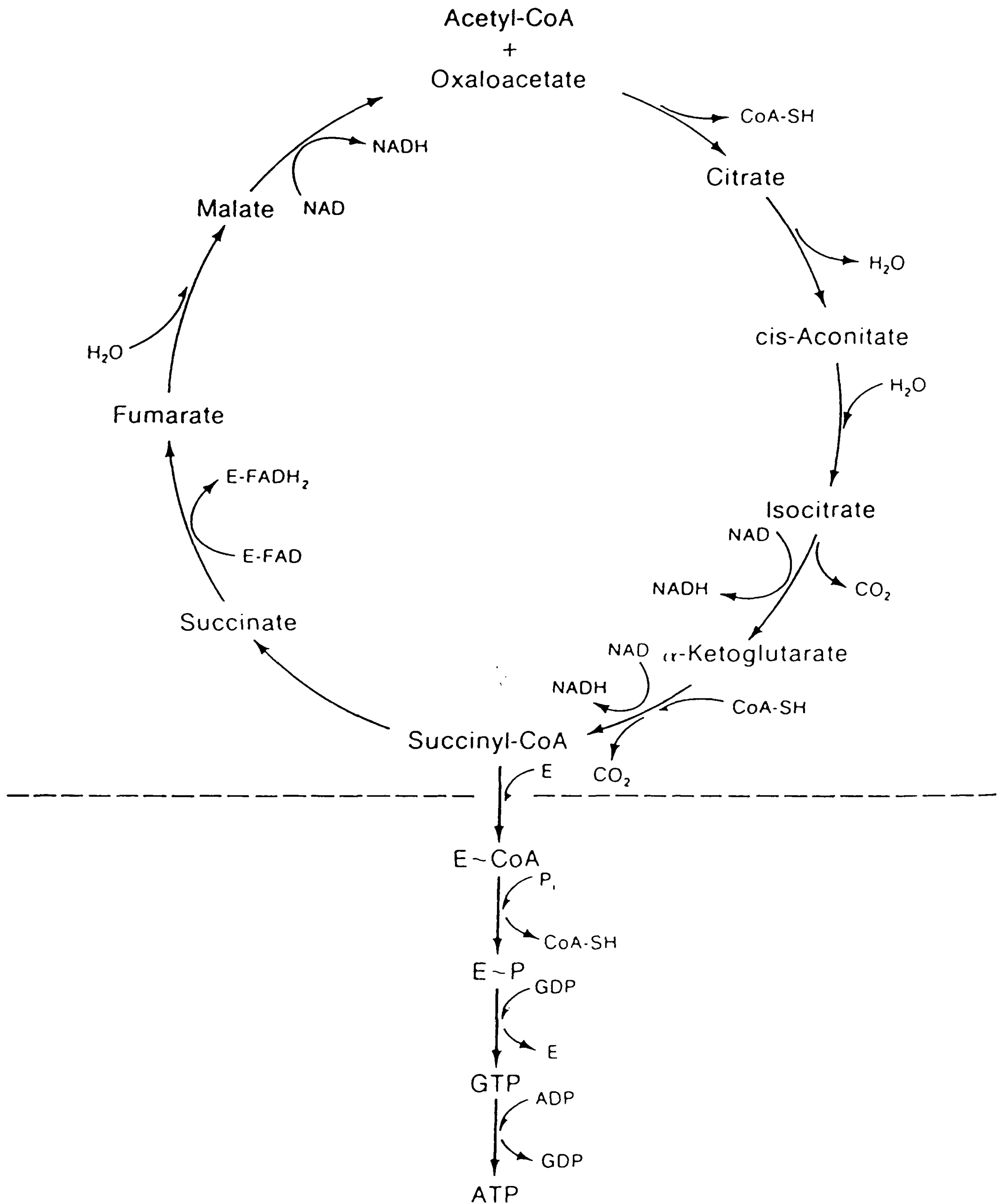
Brosnan and Fitz, 1971). Within the mitochondria, the acetyl-CoA produced from long chain fatty acyl-CoA in the ß-oxidation pathway (Figure 1.3) is further oxidised in the TCA cycle (Figure 1.4 and Section 1.2.4).

Oxidation of fatty acids by cardiac muscle results in an increase in oxygen consumption (Challoner and Steinberg, 1966). However, oxygen consumption is increased by only 13% in isolated rat hearts perfused with 1.2mM palmitate bound to 3% albumin compared to hearts perfused with glucose (Oram et al., 1973). Opie (1991) reported a similar value. This agrees with the theoretical increase expected of a shift from oxidation of glucose with a P/O ratio of 3.2 to oxidation of fatty acid with a P/O ratio of 2.8. Therefore, for the same rate of ATP synthesis, more oxygen must be consumed when fatty acids are used as substrate and Opie (1975), reported that the complete oxidation of fatty acids requires 11% more oxygen per C unit compared with the oxidation of glucose. However, Challoner and Steinberg (1966) found that perfusion with high concentrations of palmitate increased oxygen consumption by 40% and Mjos (1971) reported an increase of 25% during perfusion with lipid and heparin in the dog. The discrepancy between the actual and theoretical values for oxygen consumption in the presence of fatty acids has led to the suggestion that excess lipid uptake results in an "oxygen wasting" effect in which ATP and oxygen are used in increased esterification of fatty acids into triglycerides (Ball, 1965), uncoupling of oxidative phosphorylation (Borst *et al.*, 1962) and heat dissapation (Mjos and Kjekshus, 1971).

1.2.4 The Tricarboxylic acid (TCA) cycle and oxidative phosphorylation

Further oxidation of the acetyl-CoA produced by ß-oxidation and by the PDH complex takes place within the inner membrane of mitochondria where it enters the TCA cycle on condensation with oxaloacetate to form citrate (Figure 1.4). Oxidation in the TCA cycle produces GTP, carbon dioxide and reduced forms of

Figure 1.4 The Tricarboxylic Acid Cycle



nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$).

Reoxidation of NADH and $FADH_2$ also takes place in the mitochondrial inner membrane via electron transport. The transfer of a pair of electrons from NADH or $FADH_2$ to the final acceptor, molecular oxygen, completes an electrochemical proton gradient across the mitochondrial inner membrane. Coupling of the energy available in this gradient to that required for the synthesis of ATP from ADP and inorganic phosphate occurs via the ATP synthetase enzyme. This protein complex is located in and on the inner membrane of the mitochondria. Before ATP can be used for cytosolic reactions, it must therefore be transported out of the mitochondria and this is achieved by adenine nucleotide translocase. The ATP transported is converted to creatine phosphate by creatine phosphokinase, located in the space between the two mitochondrial membranes, and ADP is liberated. The ADP re-enters the mitochondrial matrix and creatine phosphate is converted to ATP by creatine phosphokinase, at the sites of ATP hydrolysis e.g. contractile proteins.

1.3 THE EFFECTS OF ISCHAEMIA ON MYOCARDIAL FUNCTION AND METABOLISM

Both acute and sustained ischaemia result in characteristic changes to myocardial function through energy deprivation and loss of ionic homeostasis and cell structure (Hearse, 1980).

1.3.1 Cell Structure

Hearse (1980) has described, in detail, the time-dependent sequence of cellular events induced by ischaemia. One of the earliest structural alterations is cell swelling due to osmotic changes resulting from ion imbalance. This is followed by mitochondrial and t-tubule swelling.

In a study of acute ischaemia (30-40mins) induced by coronary artery occlusion in dogs, Jennings and Ganote (1974) found swollen mitochondria, decreased matrix density and deposits of amorphous densities within the matrix space. The appearance of these amorphous densities within the mitochondria has been associated with the accumulation of lipid metabolites (Feuvray and Plouet, 1981).

Longer periods of ischaemia result in contracture development, myofibrillar disruption, extensive cellular oedema and cell membrane damage. At later stages, proteins and enzymes leak from the cells (Hearse, 1980).

An important feature identified by Feuvray (1981) is that even after global ischaemia, ultrastructural changes were not homogenous indicating that the degree of ischaemia may vary within the affected area of myocardium.

1.3.2 Contractile Function

Two main changes to contractile function are evoked in response to ischaemia. Firstly, there is a rapid loss of contractile force, then as the ischaemic episode progresses, contracture develops when the ability to maintain the normal diastolic tension is lost.

1.3.2.1 Factors contributing to loss of contractile force

The possible mechanisms of acute contractile failure have been reviewed by Allen and Orchard (1987). The relationship between ATP depletion and contractile failure is unclear. It has been suggested that contractile failure develops before substantial loss of high energy phosphates (Fawaz *et al.*, 1957; Pool *et al.*, 1966) while others have found that high energy phosphate loss preceeds or parallels this event (Hearse, 1979).Other potential mechanisms which have been considered include changes to the action potential, impairment of the contractile process and loss of calcium homeostasis.

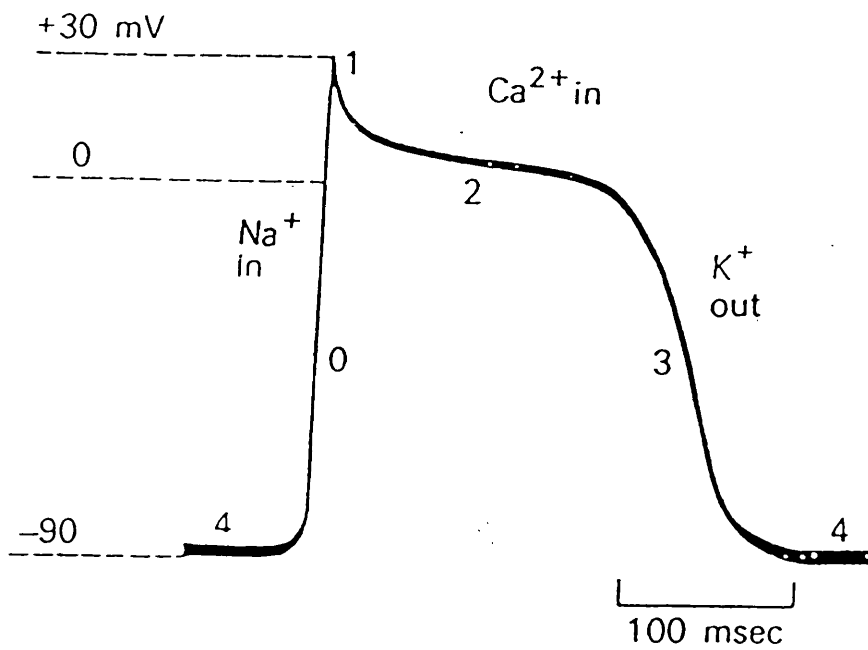
Changes in action potential configuration

Several studies have reported a positive correlation between action potential duration (APD) and the contractile amplitude in the normoxic heart (Vaughan Williams, 1959; Braveny and Sumbera, 1970; Morad and Goldman, 1973). Others have found no such correlation (Sleator et al., 1964; Sumbera, 1970; Allen, 1977). Although the reported changes in APD in both ischaemia and hypoxia are variable (Downar *et al.*, 1977), shortening of APD is a consistent finding in both conditions (McDonald and MacLeod, 1973; Vleugels *et al.*, 1980; Janse and Kleber, 1981). Metabolic inhibition (Kohlhardt and Kubler, 1975) or increased intracellular H^+ (Kohlhardt *et al.*, 1976) have been shown to cause a reduction in intracellular Ca^{++} Changes in Ca^{++} current could also contribute to APD shortening (Vleugels *et al.*, 1980). APD shortening has been associated with a reduction in the plateau (phase 2) of the action potential (Figure 1.5) and this may reflect decreased Ca^{++} entry via the slow inward current (Kholhardt and Kubler, 1975; Irisawa *et al.*, 1983). It has been demonstrated that the failure of contractility in hypoxic and ischaemic hearts is not due to a corresponding reduction of the Ca^{++} transient (Lee et al., 1988; Kihara et al., 1989; Koretsune and Marban, 1990), suggesting that contractile failure during ischaemia may be due to impairment of the Ca^{++} mediated excitation-trigger mechanism. However, other studies have not confirmed this finding (Kihara *et al.*, 1989 and have reported a decrease in the Ca^{++} transient.

A reduction in Ca^{++} release from the sarcoplasmic reticulum has been suggested as a possible mechanism (Allen and Orchard, 1983).

Potassium accumulation in the extracellular space is a common feature in ischaemia (Aksnes, 1992) and is associated with membrane depolarisation and APD shortening (Carmeliet, 1978). Vleugels *et al* (1980), suggested that an increase in K^+ outward current was the major component of APD shortening in hypoxia and this event has been linked to the activation of ATP-dependent K^+ channels (Noma

Figure 1.5 The Cardiac Action Potential Configuration



and Shibasaka, 1985; Stanfield, 1987). However, it has been shown that the changes in extracellular K^+ associated with ischaemia have only slight effects on contractility (Kavaler *et al.*, 1972) and no correlation was found between APD shortening and early contractile failure under anoxic conditions (McDonald and MacLeod, 1973). The hypothesis that contractile failure during ischaemia is a consequence of K_{ATP} channel activation due to decreased ATP levels has not been fully accepted. One reason for this has been the lack of correlation between the decrease in ATP levels and the rate and degree of contractile failure (Elliot *et al.*, 1989; Arai *et al.*, 1992). As a result, compartmentation of ATP within the cell has been postulated (Gudbjarson *et al.*, 1970; Bricknell and Opie, 1978) and inhomogeneity of ATP concentrations within or between ischaemic cells has been proposed (Nichols *et al.*, 1991; Allen and Orchard 1987).

Inorganic phosphate (P_i) and protons (H^+) have also been implicated in loss of contractilty during ischaemia. The P_i concentration rises at an early stage following the onset of ischaemia (Matthews et al., 1981; Allen at al., 1985). Theoretically, a 20% decrease in [CrP] can account for an increase of 160% in [P_i] (Allen and Orchard, 1987).

1.3.2.2 Factors contributing to contracture development

Decreased ventricular distensibility has been associated with impaired myocardial relaxation during ischaemia (McLarin *et al.*, 1973; Bourdillon *et al.*, 1983). Diastolic relaxation is achieved by restoration of the intracellular resting calcium level (0.1μM) through the activity of energy-dependent Ca^{++} ATPase (Bricknell et al., 1981). Although contracture development during ischaemia has been closely associated with the decline of ATP (Hearse et al., 1977), several hypotheses have been proposed as potential mechanisms for this event:

1) Imbalanced cytosolic calcium homeostasis due to ATP deficiency (Nayler *et al.*,

1979).

2) Increased sensitivity of contractile proteins to calcium as a result of changes to Mg^{++}, ATP and H^+ (Holubarsch *et al.*, 1982).

3) Development of rigor complexes (Hearse et al., 1977; Bing and Fishbein, 1979; Allen and Orchard, 1983).

Contracture development may further restrict coronary blood flow through subendocardial tissue exacerbating ischaemic damage (Bricknell *et al.*, 1981) and the resulting physical stress imposed on the myocytes may lead to increased membrane permeability (Ganote *et al.*, 1981). This may also contribute to reperfusion damage through defects in the glycocalyx and lipid bilayer (Frank *et al.*, 1982) caused by exposure to low extracellular calcium (Zimmerman and Hulsmann 1966; Hearse *et al.*, 1978; Rich and Langer, 1982) and decreased oxygen availability (Ganote and Kaltenbach, 1979) during the ischaemic period. Under *in vitro* conditions, contracture development is dependent on the substrate supplied (Lipasti *et al.*, 1978; Bricknell and Opie, 1984) and can be influenced by interventions which alter ATP utilisation (Nayler *et al.*, 1978).

The source of ATP production during ischaemic episodes may be important and some evidence exists to support the theory that glycolytic ATP has a specific role in maintaining diastolic relaxation and preventing contracture during ischaemia (Hearse and Chain, 1972; Bricknell *et al.*, 1981; Doorey and Barry, 1983; Weiss and Hiltbrand, 1985). Allen *et al* (1985), showed that hypoxic contracture was dependent on simultaneous inhibition of oxidative phosphorylation and glycolysis and contracture development has been shown to be lower in hearts perfused with glucose compared to hearts perfused with acetate or under glucose-free conditions (Bing *et al.*, 1975). However, whether contracture is due to low ATP or high calcium availability at the myofilaments remains unclear (Nayler *et al.*, 1988).

Sarcoplasmic reticular dysfunction

In humans, the calcium release channel in the sarcoplasmic reticulum (SR) is impaired in chronic ischaemia (Movesesian *et al.*, 1989; Brillantes *et al.*, 1992) and

impaired SR function has been associated with post-ischaemic "stunned" myocardium (Krause *et al.*,1989). Extensive swelling and distortion of the SR has been reported during 20-30 minutes of ischaemia (McCallister *et al.*, 1978; de Leiris and Feuvray, 1977) and this damage has been attributed to decreased Ca^{++}ATPase activation as a result of decreased high energy phosphate cycling and acidosis (Mandel, 1982; Krause and Hess, 1984). Recently, Kaplan *et al* (1992), have indicated that sarcoplasmic reticular dysfunction is due to reduced Ca^{++} uptake rather than release. Calcium uptake into the SR has been shown to be impaired during ischaemia (Dawson *et al.*, 1980). Sarcoplasmic reticular dysfunction has been implicated in the increase of intracellular calcium in ischaemic myocytes (Krause and Hess, 1984) and interventions which deplete calcium stores in SR have been shown to reduce the rise in $[Ca^{++}]_i$ which occurs during ischaemia (Schoutsen *et al;* 1989; Northover, 1991). Increased calcium at the level of the myofilaments (Lodge and Gelbland, 1988) and activation of Ca^{++} ATPases (Barry *et al.*, 1987) may also increase the rate of ATP depletion leading to rigor contraction.

Intracellular calcium

The level of free Ca^{++} within the cell, at any time, is the net result of Ca^{++} influx, Ca^{++} efflux, the capacity for Ca^{++} storage in organelles and the buffering capacity of cytosolic proteins.

Harding and Poole-Wilson (1980), have suggested that contracture development during hypoxia is independent of a net gain in calcium and Bourdillon and Poole-Wilson (1982), found no net increase in uptake or efflux of calcium during the ischaemic phase in ventricular tissue. Others have reported that total cystolic calcium rises during ischaemia (Marban *et al.*, 1987; Steenbergen *et al.*, 1987) suggesting that the increase in $[Ca^{++}]_i$ observed during ischaemia may represent release from an intracellular source. An increase in free intracellular calcium has been reported to occur as early as 1-2 minutes after the onset of global

ischaemia (Lee *et al.*, 1987). Allen and Smith (1985), have shown that during hypoxia, contracture precedes a rise in $[Ca^{++}]_i$ and these results are supported by studies using aequorin (Cobbold and Bourne, 1984). However, interpretation of changes in $[Ca^{++}]_i$ from different studies requires caution since hypoxia and ischaemia may produce different effects on calcium homeostasis (Kihara *et al.*, 1989).

Sodium/calcium exchange

More recently, increased calcium uptake related to Na^+ accumulation under ischaemic conditions has been reported (Tani and Neely, 1989, 1990). Under normal conditions, Na^+/Ca^{++} exchange is thought to promote calcium efflux (Mullins, 1979; Caroni and Carafoli, 1983;). In normoxia, the maximal calcium transport velocity of this process is 30-fold higher compared to the sarcolemmal Ca^{++}/Mg^{++} ATPase (Caroni and Carafoli, 1981) but the activity of Na^+/Ca^{++} exchange may be reduced in ischaemia (Bersohn *et al.*, 1982). The increase in $[Na^+]_i$ observed during ischaemia may also be a consequence of increased H^+/Na^+ exchange coupled with decreased Na^+/K^+ATPase activity (Ladzunski *et al.*, 1985; Tani and Neely, 1990). Lazdunski *et al* (1985), have proposed that a combination of Na^+/H^+ and Na^+/Ca^{++} exchange would result in Ca^{++} influx with subsequent Ca^{++} overload on reperfusion.

Agents which inhibit Na^+ channels (Takeo *et al.*, 1989) or otherwise prevent Na^{++} overload (Boddeke *et al.*, 1989), have been shown to have beneficial effects under ischaemic conditions.

1.3.3 Metabolic function in ischaemia

Although the metabolic consequences of anoxia and hypoxia can be fundamentally different from those of ischaemia (Hearse, 1980), these conditions have proved useful in delineating some of the effects of ischaemia.

The principal difference between ischaemia and anoxia/hypoxia is the maintenance of perfusion and substrate supply throughout the experimental period. This prevents accumulation of metabolic products and, in particular, removes the inhibitory effect of lactate (i.e. H^+) on phosphofructokinase. Consequently, under conditions of anoxia and hypoxia, glycolysis contributes to ATP production, while in ischaemia the activity of this metabolic pathway will be progressively inhibited.

The principal events resulting from ischaemia are 1) deprivation of oxygen, 2) deprivation of substrate supply and 3) the inability to remove the products of cellular metabolism. As already mentioned the effects of ischaemia on cell metabolism and structure are critically dependent on the duration of the ischaemic insult (Hearse, 1980) and it is well recognised that a time-dependent transition from reversible to irreversible cell damage occurs during ischaemia. In the absence of oxygen, electron transport and oxidative phosphorylation are arrested and the cytosolic and mitochondrial levels of ATP decrease.

Glycolysis in the ischaemic heart

Under anaerobic conditions, it has been estimated that, at maximum, glycolysis could supply 20% of the ATP required for normal cell function (Opie, 1968). Anoxia accelerates glucose utilisation (Neely and Morgan, 1974) but in ischaemia, although initially stimulated, the glycolysis pathway is inhibited at the level of phosphofructokinase (Kubler and Spiekerman,1970) and glyceraldehyde-3-phosphate dehydrogenase (Rovetto *et al,* 1975) by H^+ accumulation . This inhibitory effect reflects a reduction in flux through the glycolytic pathway, not a lower rate of glucose transport, and cannot be reversed by increased glucose and insulin. (Rovetto *et al.*, 1973).

The association of enzyme release with infarcted myocardium has been well described (Hearse, 1977; Lott and Stang, 1980). Under in vitro conditions, enzyme release has been shown to be affected by substrate in that release is lower in glucose perfused hearts compared to hearts perfused with free-fatty acid (deLeiris and Opie, 1978). It is thought that the positive effects of glucose and insulin in the

ischaemic heart are related to improved carbohydrate utilisation and a reduction in accumulated lipid intermediates (Farah and Alousi, 1981). ATP production from increased glycolytic flux (Opie and Bricknell, 1979) may diminish the effects of free-fatty acid (Kurien and Oliver, 1970; Weishaar *et al.*, 1977) and acyl CoA accumulation (Shug *et al.*, 1975). It has recently been suggested that the protective effect of glucose may be affected by dietary lipids (Moreau and Chardigny 1991) although the mechanism by which glucose exerts its protective effect remains under debate.

Experimental evidence indicates a protective role for glucose in the ischaemic myocardium (Opie and de Leiris, 1979; Opie and Bricknell, 1979). It has been suggested that ATP produced in the cytoplasm from glycolysis may have a special role in the maintenance of membrane integrity and electrical stability (Bricknell and Opie, 1978a). Thus, glucose has been shown to protect against arrhythmia development in coronary ligated dog hearts (Surer *et al.*, 1976; Russell and Oliver., 1979.) *in vivo* and *in vitro* (Dennis *et al.*, 1979) and on reperfusion (Bricknell and Opie, 1978b). Elevation of extracellular glucose has also been shown to prevent shortening of action potential duration (APD) induced by inhibition of oxidative phosphorylation or hypoxia (MacLeod and Daniel, 1965; MacLeod and Prasad, 1969). APD shortening through glycolytic inhibition by 2-deoxyglucose is not affected by increased glucose indicating that increased glycolytic flux may be the main contributory factor in the protective effect of exogenous glucose (Nakamura et al., 1989).

Intracellular acidosis

The effect of acidosis in conditions of oxygen deprivation may be two-fold. Through its negative inotropic effect, acidosis may conserve ATP and exert a protective effect (Bing *et al.*, 1973) or it may decrease glycolytic production of ATP and enhance ischaemic damage. Protons accumulating in the cell arise from a number of sources including adenine nucleotide degradation, accumulation of carbon dioxide from residual oxidative metabolism, decarboxylation reactions and

triglyceride cycling (Opie, 1976; Gevers, 1977). During sustained ischaemia, acidosis may cause activation of lysosomal hydrolases and lipoprotein lipases which can contribute to loss of cell structure (Ricciuti, 1972; Weglicki *et al.*, 1974).

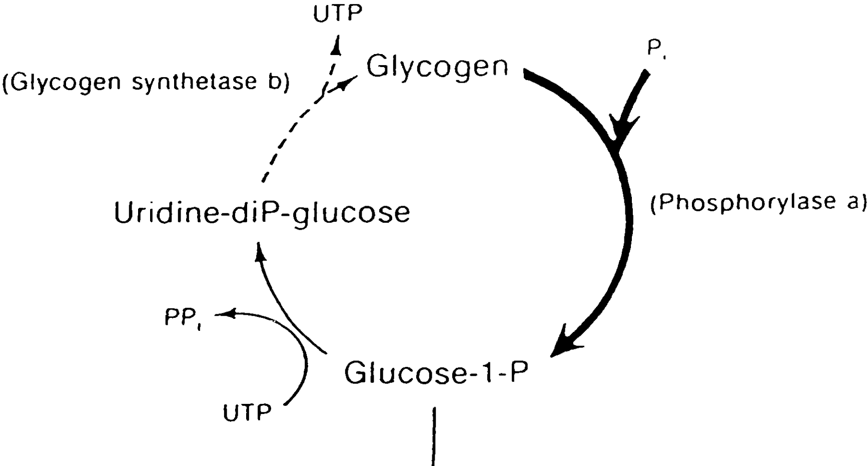
Changes in intracellular pH are dependent on the degree and duration of ischaemia. Nuclear magnetic resonance studies have shown that, in global ischaemia and coronary artery occlusion, intracellular pH changes by about 1pH unit (Pieper *et al.*, 1980; Flaherty *et al.*, 1982; Malloy *et al.*, 1986). Measurement of extracellular pH in ischaemic hearts have reported changes of 0.3-0.7pH units during transient and partial ischaemia (Momomura *et al.*, 1985; Ichihara *et al.*, 1986). However, studies in cardiac muscle have shown that changes in extracellular pH are not representative of the change in intracellular pH. Ellis and Thomas (1976) have shown that the $[pH]_i$ changes by 0.2pH units for 1 unit change in $[pH]_o$, indicating that studies using extracellular acidosis to investigate the effects of ischaemia may significantly overestimate the effect on intracellular pH.

Although the capacity of glycolysis for ATP production in relation to the requirements of a normal cell may be limited under ischaemic conditions, cellular demands on energy reserves are markedly reduced by the subsequent decrease of contractile activity in the ischaemic area. In addition, collateral flow and incomplete occlusion will result in ischaemic conditions of varying intensity which will affect the degree of inhibition of glycolytic metabolism.

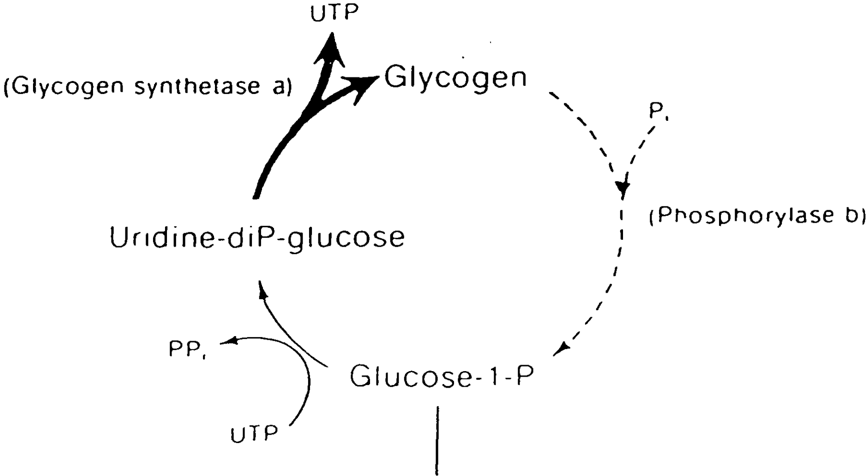
Glycogen metabolism in the ischaemic heart

Glycogen represents a stored form of glucose that can be readily mobilised in heart muscle. It is present in the form of granules localised around the mitochondria, interfibrillar sarcoplasm and subsarcolemmal cisternae of the sarcoplasmic reticulum. The distribution of glycogen within the myocardium is not homogenous and levels in endocardial tissue are twice that found in the epicardium (Jedieken, 1964).

Figure 1.6 The Enzymatic Control of Glycogen Metabolism



PHOSPHORYLATED ENZYMES



DEPHOSPHORYLATED ENZYMES

Glycogen metabolism is controlled by the enzyme, phosphorylase, which exists in phosphorylated and dephosphorylated forms. Glycogen phosphorylase activity is under hormonal as well as intracellular control and regulates glycogen mobilisation and synthesis in conjunction with glycogen synthetase. When both enzymes are phosphorylated (e.g. as a result of cAMP elevation and activation of protein kinase A), glycogen synthetase is present in the inactive *b* form and phosphorylase is activated (Figure 1.6). Under these conditions, glycogen synthesis is inhibited and mobilisation is accelerated. When both enzymes are dephosphorylated, glycogen synthesis is accelerated and mobilisation is inhibited. The significance of phosphorylation-dephosphorylation conversion of enzyme activity in metabolic control mechanisms has been reviewed by Chock *et al.*, (1980).

The potential for glycogen to exert a protective effect during ischaemia is derived from the anaerobic synthesis of ATP through maintained glycolytic flux despite inadequate supply of exogenous substrate. In theory, utilisation of glycogen would be advantageous since metabolism of 1 mole of glucose via glycogenolysis would yield 3 ATP molecules compared to 2 ATP molecules from 1 mole of glucose supplied by uptake. Several studies have demonstrated glycogen utilisation in the ischaemic, hypoxic and anoxic myocardium (Neill *et al.*, 1963; Weissler *et al.*, 1968; Lolley *et al.*, 1979; Grover *et al.*, 1981). This may be caused by elevated cAMP and/or Ca^{++} mediated processes (Rovetto *et al.*, 1975) and activation of glycolytic enzymes (Rovetto *et al.*, 1973). Activation of phosphorylase has been observed in the ischaemic myocardium (Wollenberger and Krause, 1968; Kubler and Spiekerman, 1970; Sakai and Abiko, 1981) but phosphorylase activity can also be increased by inorganic phosphate and AMP as well as reduced ATP and glucose-6-phosphate concentrations (Opie, 1968). In addition, changes to metabolism in response to ischaemia have been observed in the absence of elevated cAMP (Podzuweit et al., 1978). Therefore, the role of cAMP in glycogenolysis during ischaemia is still unclear.

Although the ability of glycogen metabolism to alter the pathogenesis of the severely ischaemic area may be limited by glycolytic inhibition, it may be more important in the so called "border zone" where some degree of flow and oxygenation may continue. Rovetto et al., (1973), have reported that glycogen utilisation is much faster in anoxic rat hearts where perfusion was maintained compared to flow restricted (ischaemic) hearts and hearts with increased glycogen stores have been shown to have improved tolerance to conditions of oxygen deprivation (Scheur and Stezoski, 1970; Hewitt *et al.*, 1973). However, the conclusions on the contribution of glycogen to ischaemic energy production have been drawn from models using ischaemia, hypoxia and anoxia and require careful interpretation since anaerobic potential differs greatly in these models (Hearse 1980).

Lipid metabolism in the ischaemic heart

Ischaemia is associated with altered lipid metabolism (Jodalen *et al.*, 1985) and the potential adverse effects of accumulated fatty acids and other lipid intermediates in the ischaemic myocardium have been widely investigated (Liedtke, 1981; Katz and Messineo, 1981; Corr *et al.,* 1984; Hutter and Sobol, 1992).

A rapid elevation of mitochondrial $NADH/NAD^+$ and $FADH_2/FAD$ ratios (Neely and Morgan, 1974) inhibits fatty acid oxidation at the level of NAD-linked ß-hydroxyacyl-CoA dehydrogenase (Moore, 1985), resulting in increased levels of NEFA, acyl CoA and acylcarnitine (Opie, 1979; van Bilsen *et al.*, 1989; Hutter and Sobol, 1992). In severely ischaemic myocardium, NEFA levels can increase 6-7 fold (van der Vusse *et al.*, 1982) while, under ischaemic conditions *in vivo* , acylcarnitine levels have been reported to increase 18 fold (Liedtke *et al.*, 1978). Additionally, phospholipases are activated (Katz and Messineo, 1981) and lysophospholipid levels are raised (Corr *et al.*, 1982). In patients with myocardial ischaemia, plasma fatty acid levels do not generally increase until 1-2 hours after the onset of symptoms (Vetter *et al.*, 1974). However, tissue levels of free fatty acid can change, independently of plasma levels, through catecholamine mediated

lipolysis and increased myocardial extraction of free fatty acid from the blood (Simonsen and Kjekshus, 1978; Vik-Mo *et al.*, 1979, 1981).

Free fatty acids and their metabolites have been associated with a number of effects which could contribute to ischaemic damage. They are amphiphilic and have been implicated in a number of membrane dysfunctions (Katz and Messineo, 1981; Corr *et al.*, 1984). High concentrations of fatty acids cause uncoupling of mitochondrial respiration (Borst *et al.*, 1962) and the velocity and capacity of mitochondrial calcium uptake is decreased (Piper *et al.*, 1983). Increased endogenous levels of NEFA may exert negative inotropic effects (Henderson *et al.*, 1970), alter mitochondrial permeability (Wotzak, 1974) and accelerate glycogenolysis during ischaemia (Cowan and Vaughan Williams, 1980). NEFA increase cardiac oxygen consumption (Mjos, 1971) and shorten action potential duration (Cowan and Vaughan-Williams, 1977). Action potential shortening is an early event in ischaemia (McDonald and MacLeod, 1973) and has been associated with the rapid loss of contractile function in the ischaemic myocardium (Nayler *et al.*, 1979). Elevated free fatty acids can also inhibit glucose oxidation (Renstrom *et al.*, 1990) which may affect regions of the ischaemic myocardium during ischaemia (Piper and Das, 1986) and reperfusion (Lopashuk *et al.*, 1990) through impaired glycolysis and increased oxygen consumption.

NEFA accumulation has been observed in glucose-perfused hearts subjected to no-flow ischaemia (van Bilsen et al., 1989). Under these conditions, tissue levels of NEFA increased between 30 and 60 minutes (Prinzen *et al.*, 1984). However, the increase in fatty acid intermediates during no-flow ischaemia was minimal (Neely *et al.*, 1979). These observations indicate that endogenous lipid contributes to NEFA accumulation during no-flow ischaemia. While levels of acylcarnitine and acylCoA are not increased under no-flow ischaemic conditions (Poulson *et al.*, 1984), NEFA and fatty acid metabolites increase under conditions of reduced flow and may exert profound effects on the ischaemic cell (Corr et al., 1984). In dog hearts *in vivo*, coronary occlusion resulted in increased levels of

acyl CoA and acylcarnitine within 15 minutes of ischaemia (Suzuki *et al.*, 1981) indicating that the increase in fatty acid metabolites is a rapid event. Mitochondrial accumulation of long chain acylCoA and increased cytosolic levels of acylcarnitines (Idell-Wenger *et al.*, 1978; Moore *et al.*, 1984) are most pronounced in ischaemic hearts perfused with exogenous NEFA (Shug *et al.*, 1978; Neely *et al.*, 1979). In addition, high levels of plasma NEFA increase tissue accumulation of lipid intermediates during ischaemia (Liedtke *et al.*, 1978).

Lipid intermediates

Long chain acyl-CoA and acylcarnitines have been identified as the major metabolites of fatty acid metabolism and have been shown to affect a number of intracellular mechanisms. Mitochondrial respiration is inhibited by high concentrations of long chain acylcarnitines and acyl-CoA (Pande and Blanchar, 1971; Wood *et al.*, 1977). Palmitoyl carnitine alters Ca^{++} pump activity (Barry *et al.*, 1978; Pitts *et al.*, 1978) and enhances Ca^{++} release (Pitts *et al.*, 1978; Messineo *et al.*, 1982) by the sarcoplasmic reticulum (SR). These effects are dependent on the carbon chain length and concentration of acylcarnitine (Owens *et al.*, 1982). Low concentrations of palmitoyl carnitine (5-50μM) stimulate Ca^{++} ATPase and Ca^{++} binding in the SR and high concentrations (50-200μM) inhibit these activities (Adams *et al.*, 1979). Palmitoyl carnitine may alter contractile force through inhibition of Na^{+}/Ca^{++} exhange and increased Ca^{++} entry (Inoue and Pappano, 1983). The effect of palmitoyl carnitine on Na^{+}/K^{+}ATPase activity is unclear (Adams *et al.*, 1979; Osornio-Vargas *et al.*, 1981; Owens *et al* 1982) but inhibition of sodium pump activity by palmitoyl carnitine has recently been demonstrated in myocytes (Tanaka *et al.*, 1992). The activity of palmitoyl carnitine is enhanced at pH values (6.7) consistent with ischaemic conditions and the electrophysiological effects of palmitoyl carnitine are not produced by palmitic acid or carnitine (Corr *et al.*, 1984).

Inhibition of adenine nucleotide translocase by long-chain acyl CoA has been reported (Shug *et al.*, 1975; Shrago, 1976; La Noue and Schoolwerth., 1979). In

contrast, short-chain acyl CoA (Woldegiorgis *et al.*, 1981) and long-chain acylcarnitine (Shrago *et al.*, 1976) do not inhibit this enzyme indicating a specific interaction which may be receptor-mediated (Woldegiorgis *et al.*, 1982). During prolonged ischaemia, acylcarnitines may leak from the cytosol to the extracellular space (Shug *et al.*, 1978), and the effects induced by extracellular compared to intracellular acylcarnitine may differ. Experimental evidence shows that the increase in acyl-CoA is largely mitochondrial but mitochondrial membrane permeability can be increased by high concentrations of acyl CoA (Wojtczak, 1974). The proximity of increased fatty acid intermediates to intracellular membranes during ischaemia has not been elucidated and although autolysis and cell damage can occur in the absence of fatty acid intermediate accumulation (Neely *et al.*, 1979), the diverse effects of these amphiphilic moieties indicate the potential contribution of impaired lipid metabolism to ischaemic cell damage.

Catecholamines and cAMP

Under ischaemic conditions, catecholamine release has a major influence over the metabolism of lipid and glycogen. Since the early report, by Forssman *et al* (1952) that myocardial ischaemia is associated with increased catecholamine levels in the blood, it has been confirmed in numerous studies that this event leads to exacerbation of ischaemic damage (Raab, 1963). This is due to stimulation of adenylate cyclase activity and the subsequent elevation of cAMP (Wollenberger *et al.*, 1967). In early ischaemia, the elevation of plasma NEFA may be a consequence of raised catecholamine levels (Oliver *et al.*, 1968) and Opie *et al.* (1971) have suggested that adrenergic stimulation leads to increased lipid metabolism. This causes elevated myocardial oxygen consumption (Mjos 1971) and thus, increases the ischaemic area (Kjeksus and Mjos 1972). Summanson and Kjeksus (1978), suggested that the free fatty acid concentration was not of primary importance in affecting myocardial oxygen consumption unless accompanied by catecholamine stimulation. The true relationship between catecholamines and changes in lipid levels is unclear however, because the time of increase of plasma

catecholamines following ischaemia does not correlate with the increase in plasma NEFA levels (Velter *et al.*, 1974). In addition, Stam *et al* (1987) have proposed that increased lipolysis during ischaemia results indirectly from simultaneous glycolytic stimulation (Schoonderwoerd *et al.*, 1987).

Despite the low oxidation capacity of ischaemic tissue (Schuer and Brachfield., 1966), it has been reported that free-fatty acid uptake is increased in ischaemic tissue in the presence of catecholamines (Vik-Mo *et al.*, 1979). This contrasts with the finding that NEFA uptake is reduced in ischaemic tissue (van der Vusse *et al.*, 1982). The fate of these fatty acids is thought to be triglyceride esterification (Schuer and Brachfield, 1966) and it has been found that triglyceride lipolysis and re-esterification is enhanced in ischaemic tissue (Hough and Gevers 1975; Brownsey and Brunt, 1977).

Triglycerides

Recycling of free fatty acids in the triglyceride pool may contribute to the energy wasting effects of free fatty acids (Vik-Mo and Mjos, 1981). Under *in vitro* conditions, it has been shown that degradation of endogenous lipids such as triglycerides and phospholipids can increase myocardial levels of NEFA in the absence of exogenous lipid substrate (van Bilsen *et al.*, 1989). The release of glycerol and the increased tissue level of glycerol-3-phosphate in ischaemic tissue is thought to reflect enhanced activity of the NEFA-triacylglycerol cycle which maintains an equilibrium between the rate of hydrolysis and synthesis of triacylglycerol (Vik-Mo and Mjos, 1981). This cycle is associated with ATP consumption required for activation of NEFA to form acyl-CoA for subsequent re-esterification. The mechanisms involved in the regulation of this cycle, under ischaemic conditions, are unclear. Karwatowska-Krynska and Beresewicz (1983) suggested that glycerol production during hypoxia was the result of endogenous catecholamine release but it has been reported that the increase in glycerol output during hypoxia is not sensitive to ß-adrenergic blockade (Larsen *et al.*, 1989). More recently, Myrmel *et al* (1991) have concluded that increased fatty acid cycling

is the result of mass action by increased levels of glycerol-3-phosphate produced during increased glycolysis.

Lipase activity

Ischaemic conditions favour increased lipolytic activity in the myocardium. , Increased lipolysis due to release of endogenous catecholamines and increased lipase activation during ischaemia has been reported (Vik-Mo and Mjos, 1981; Heathers and Brunt, 1985). A hormone-sensitive lipase activated by catecholamines has been identified in adipose tissue (Belfrage *et al.*, 1984) and a similar lipase is thought to be present in heart (Christian *et al.*, 1969; Goldberg and Khoo, 1985). In addition, AMP induced stimulation of myocardial triacylglycerol lipase activity has been reported (Schoonderweoerd *et al.*, 1987). Acyl-CoA accumulation will also promote fatty acid esterification (Neely and Moran, 1974; Liedtke, 1981).

Lipolysis of endogenous triacylglycerols has been shown to be dependent on glycolysis (Schoonderwoerd *et al.*, 1987). This has led to the conclusion that increased lipolysis in the presence of isoprenaline is the result of increased glycolytic flux (Clark and Patten, 1984) and not cAMP-dependent lipase activation. The mechanism underlying increased lipolysis during ischaemia remains unclear.

1.4 CLINICAL TREATMENT OF MYOCARDIAL ISCHAEMIA

The clinical treatment of cardiac ischaemia has been aimed at increasing the supply of oxygen and substrate and/or reducing the metabolic requirements of the heart. The first discovery of an effective treatment for angina was by Brunton in 1867, when the beneficial effects of nitrates were described. These compounds relieved the restriction of flow through the coronary circulation by coronary vasodilatation (Hollmann 1963). Reducing metabolic demand was achieved by slowing the heart rate or decreasing the force of contraction in addition to reducing

preload and afterload by decreasing peripheral vascular resistance. This led to the development of ß-adrenoceptor blocking agents (Black and Stephenson, 1962) and drugs which effectively reduced calcium entry into cardiac and vascular cells (Fleckenstein 1977).

A new direction in the pharmacological treatment of ischaemia has evolved with the advent of putative metabolic modulators. Direct intervention in cardiac metabolism has been attempted with the discovery of carnitine palmitoyl transferase 1 inhibitors which alter myocardial oxygen consumption by diverting metabolism from free fatty acid towards glucose utilisation. The beneficial effects of oxfenicine (Higgins *et al.*, 1980) and phenylalkyloxirane carboxylic acid (POCA) have been already been described (Wolf *et al.*, 1981).

1.5 AIMS OF THIS THESIS

It is clear that much remains unknown about the functional and metabolic consequences of ischaemia in the myocardium. The studies described in this thesis were therefore directed:

1) To examine functional and metabolic changes induced by ischaemia in cardiac tissue by a comparison of different experimental models.

2) To investigate metabolic interventions which may directly modify the effects of cardiac ischaemia.

The primary purpose of this work is to evaluate the potential of reducing the effects of ischaemia by altering metabolism within the myocardial cell. The introduction (Chapter one) has highlighted the existence of metabolic differences in the response to ischaemia dependent on the method used to induce oxygen deprivation. Chapter two describes the methods and experimental techniques used and Chapter three uses these methods in order to identify an *in vitro* perfused heart model suitable for this evaluation. Chapter four demonstrates the consequences of impaired glucose metabolism on the functional and metabolic responses to

ischaemia and hypoxia and examines the effects of palmitate on cardiac function during ischaemia. Chapter five describes the effects of ranolazine, a novel anti-ischaemic agent, on the ischaemic myocardium and investigates the possible mechanisms of action of this drug. The recently described ATP-dependent K^+ channel has been associated with the myocardial response to ischaemia. Chapter six examines the effects of altering the activity of these channels in the myocardium and addresses the question of their contribution to the contractile effects of ischaemia.

The potential for interventions at a metabolic level in the treatment of cardiac ischaemia are discussed in Chapter seven.

CHAPTER TWO

MATERIALS AND METHODS

2.1 BIOCHEMICALS AND CHEMICALS

All chemicals and biochemicals were of analytical standard and, unless otherwise stated, were obtained from Fisons Ltd, Loughborough, U.K., BDH Ltd, Glasgow, U.K., Sigma Chemical Co., Ltd, Dorset, U.K., Aldrich Chemical Co., Ltd, Dorset, U.K., Boehringer Ltd, Sussex, U.K. Radiochemicals were obtained from Amersham International plc, Buckinghamshire, U.K. Gases were supplied by BOC Ltd, Glasgow, U.K.

Enzymes

Arylamine acetyltransferase (EC 2.3.1.5), carnitine acetyltransferase (2.3.1.21), creatine kinase (EC 2.7.3.2), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glutamine-pyruvate aminotransferase (EC 2.6.1.15), hexokinase (EC 2.7.1.1), L-lactate dehydrogenase (EC 1.1.1.27), luciferase (EC 1.13.12.7), pyruvate dehydrogenase (PDH) complex (EC 1.2.4.1/ 2.3.1.12/ 1.8.1.4), PDH phosphate phosphatase (EC 3.1.3.4).

2.2 EXPERIMENTAL ANIMALS

Female guinea-pigs (Dunkin Hartley), in the weight range 200-400 g, supplied by Harlan Porcellus, Sussex U.K., were used in all studies.

2.3 METHODS

2.3.1 The Langendorff perfused heart preparation

The method was a modification of the isolated perfused heart preparation described by Langendorff (1895).

Female guinea-pigs (Dunkin Hartley, 200-400g) were killed by cervical

dislocation. The thoracic cavity was opened and the excised heart was transferred into ice-cold heparinised physiological salt solution (pH 7.4) of the following composition (mM) : NaCl 118.4, KCl 4.7, $CaCl_2$ 1.9, $NaHCO_3$ 25, $MgSO_4$ 1.2, Glucose 11.7, NaH_2PO_4 1.2, Heparin 1unit/ml and aerated with 95%O_2, 5%CO_2. The oxygen tension of the equilibrated perfusate at 37^{0}C was measured using a blood gas analyser (Corning168) and was within a range of 450-650mmHg.

Using fine dissecting scissors, extraneous tissue was carefully removed and the aorta was cannulated using an intravenous cannula (size 5FG, Portex Ltd, Kent, U.K.). The cannulated heart was attached to the outlet of a glass perfusion chamber (10ml volume). This chamber was enclosed in a water jacket which was thermostatically heated to 37^{0}C using a water circulator (Churchill Instrument Co. Ltd, Middlesex, U.K.). The perfused heart was also enclosed within a humidified semi-sealed glass water jacket heated to 37^{0}C (Figure 2.1a).

Each heart was retrogradely perfused through the aorta with heparin-free physiological salt solution pre-heated to 37^{0}C. The perfusion rate was controlled using a peristaltic pump (Gilson, France) and the perfusion pressure was recorded using an in-line pressure transducer (type PDCR 75, Druck Ltd, Leicestershire, U.K.) located immediately above the aortic cannula. Each heart was perfused for an initial 30 minute equilibration period during which the perfusion flow rate was increased to a perfusion pressure of around 50mmHg. This resulted in flow rates of 9 ± 2ml/min.

Electrical pacing

In electrically-paced preparations,transmural stimulation (1ms duration, 12V) was delivered through two platinum electrodes attached to the ventricle walls. The stimulation frequency was set at 25% above the spontaneous heart rate using an electronic stimulator (type S88, Grass Instruments, Massachusetts, USA.). In unpaced preparations, each heart was allowed to beat at the spontaneous heart rate. The relative heart rates in unpaced and paced hearts are shown in Chapter 3.

Mechanical measurements

A ligature was attached to the ventricular apex of each heart and this was connected, through a pulley system, to an isometric tension transducer (Dynamometer UF1, Lectromed Ltd, Hertfordshire. U.K.) mounted on an adjustable clamp. Mechanical force of contraction was recorded using an electronic recorder (type MX212, Lectromed Ltd.) connected through a pre-amplifier (type 3559, Lectromed Ltd.) to the transducer. Simultaneous measurements of heart rate were made using an electronic ratemeter (type 4522, Lectromed Ltd.) which was triggered by electrical signals from the pre-amplifier. Changes in perfusion pressure, were recorded via a pressure transducer (type PDCR 75, Druck Ltd.) connected to a pressure pre-amplifier (type 4820, Lectromed Ltd.).

After the heart had been perfused for approximately 15 minutes, the tension on the ligature between the ventricular apex and the transducer was increased until the maximum force of contraction was achieved. The tension applied to each heart was equivalent to a load of around 7 g.

Collection of samples for biochemical analysis

Miniature glass filter funnels were used to direct the perfusate collected from the ventricular apex of the heart into pre-cooled glass vials (10ml volume). The samples were immediately transferred to an ice bath ($4^{0}C$) and the metabolite concentration and enzyme activities were assayed within two hours of collection.

At the end of each perfusion, hearts retained for biochemical analysis were frozen using Wollenberger clamps pre-cooled in liquid nitrogen. In this way, metabolic arrest was achieved without removing the hearts from the perfusion apparatus. The hearts were quickly weighed, wrapped in tinfoil and were stored under liquid nitrogen until the tissue metabolite content was analysed.

2.3.2 The isolated papillary muscle preparation

Guinea-pigs (Dunkin Hartley, 200-400g) were killed by cervical dislocation.

The thoracic cavity was opened and the excised hearts were transferred to a beaker of ice cold physiological salt solution of the following composition (mM); NaCl 146.5, KCl 6.0, $MgCl_2$ 1.0, Trizma HCl 7.28, Trizma base 2.74, $CaCl_2$ 2.0 Glucose 5.0 and aerated with oxygen. The pH of this solution was 7.8 at 4^{0}C and 7.4 at 37^{0}C.

Each heart was removed and placed in a petri dish containing the ice cold physiological salt solution and extraneous material was removed. Each heart was transferred to a dissecting dish and secured by dissecting pins at the ventricular apex and the left ventricle. Under a binocular microscope (Model 22995, PZO, Warsaw, Poland.), an incision was made down the side of the right ventricle. The right ventricle wall was pinned back from the heart and the papillary muscle, attached to the inner wall of the ventricle, was exposed. At one end of the muscle, the tendon (white and translucent) was cut and detached from the ventricle wall. This allowed the muscle to be lifted up and dissected out of the ventricle.

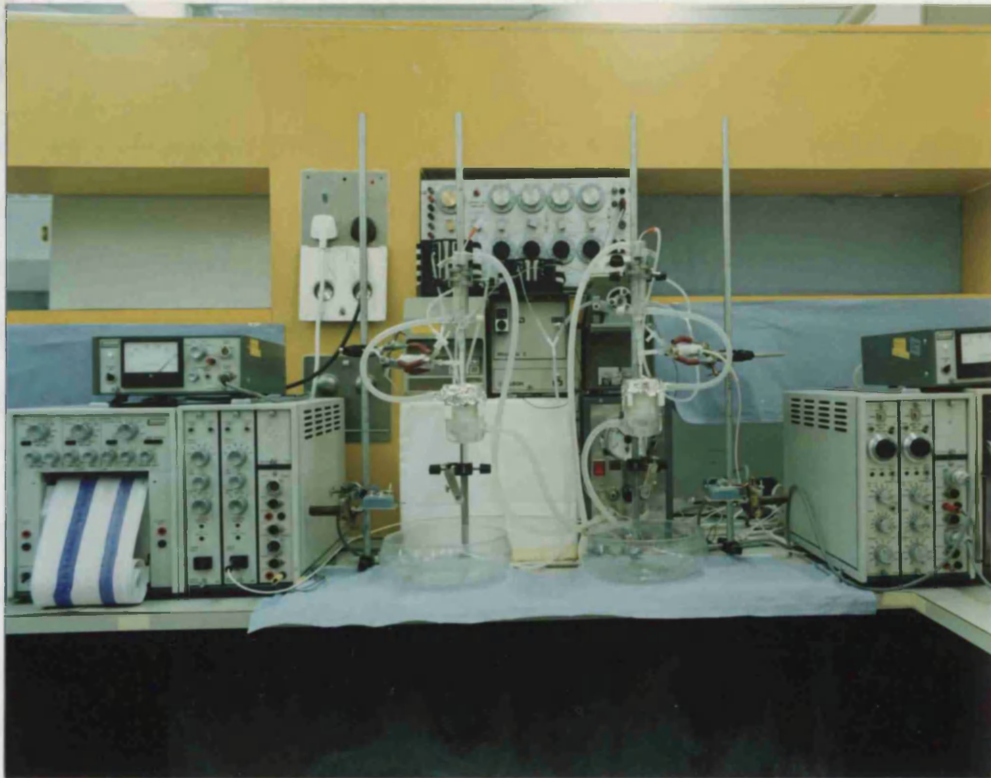
Each muscle was then transferred to a continuous flow perspex organ bath (Figure 2.1b) and anchored in position by a pin which was placed through the end of the muscle. The tissues were superfused at 5ml/min with physiological salt solution and the flow rate was controlled using a peristaltic pump (type 502, Watson-Marlow Ltd, Cornwall, U.K.). Each muscle tendon was attached to an isometric force transducer (type 875, AME, Horten, Norway.). The temperature of the perfusate was monitored by a thermistor located within the superfusion chamber and this thermistor was connected to a peltier heat controller (Firbank Electronics, Norfolk, U.K.) which maintained the temperature of the solution at 37^{0}C.

Electrical pacing

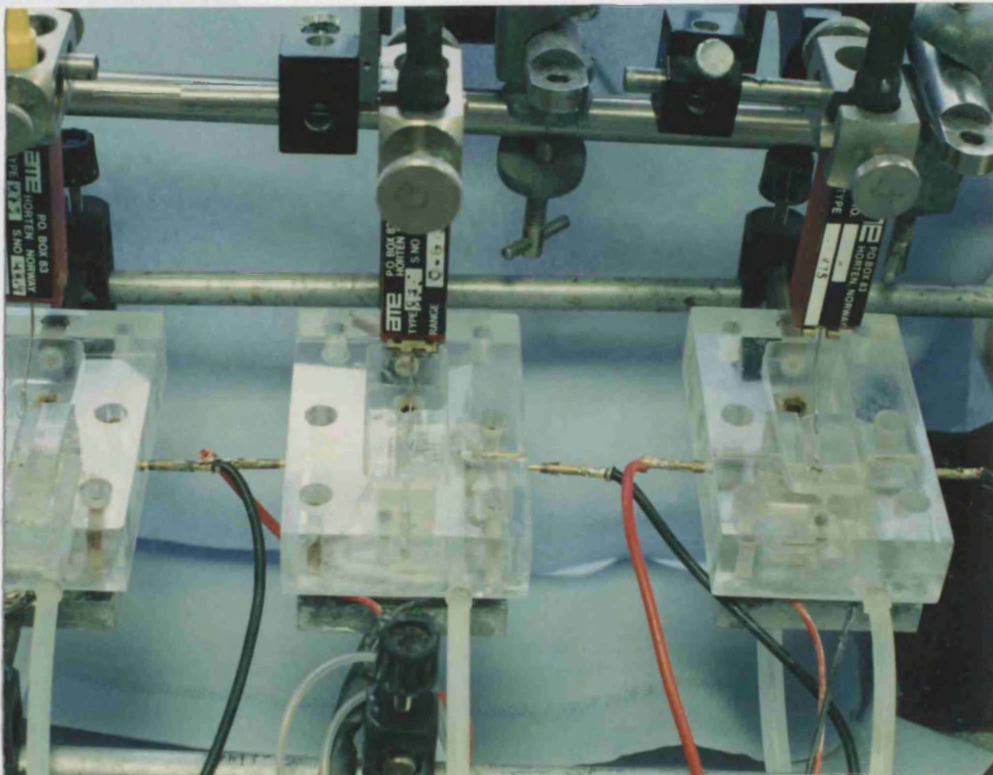
Electrical impulses (0.5ms duration, 1Hz), were delivered through two platinum wire electrodes located on either side of the organ bath. Voltage output from the electronic stimulator (Firbank Electronics) was set at zero and was gradually increased until the threshold voltage required to initiate a muscle contraction was achieved. This voltage was usually 2-3 volts. After tension had

Figure 2.1 The Langendorff heart and papillary muscle perfusion apparatus

a) The Langendorff heart perfusion system



b) The papillary muscle superfusion system



been applied to the muscle, the threshold stimulating voltage was maintained at 1 volt above threshold for the duration of the experiment.

Mechanical measurements

Mechanical force of contraction was recorded using an electronic recorder (type MX212, Lectromed Ltd.) connected through a pre-amplifier (Firbank Electronics) to the transducer. The tension between the muscle and the transducer was adjusted using a micromanipulator until the maximum force of contraction was achieved. The tension applied was equivalent to a load of around 500mg. Each tissue was allowed to equilibrate for 60 minutes prior to the start of the experiment.

Collection of samples for biochemical analysis

At the end of each perfusion, papillary muscles used for biochemical analysis were quickly transferred to a flask of liquid nitrogen. The tissues were stored under liquid nitrogen until the tissue metabolite content was analysed.

2.3.3 Tissue metabolite extraction

Perfused heart tissue.

Each heart was removed from liquid nitrogen and transferred to a stone mortar pre-cooled in frozen CO_2 pellets. Using a stone pestle cooled in liquid nitrogen, the tissue was homogenised under liquid nitrogen to form a fine powder.

Around 400mg of frozen powdered tissue was weighed in a pre-cooled tinfoil container. This sample was quickly transferred to the pre-cooled mortar and liquid nitrogen was added. The mixture was homogenised with 1ml of 6% (w/v) perchloric acid to form a dry white powder. The level of liquid nitrogen was maintained until homogenisation was complete. Using a pre-cooled metal spatula, the powder was transferred to a screw-topped cryotube (5ml capacity) which was placed in an ice bath to allow slow thawing of the sample.

When thawed, the sample was centrifuged in a bench centrifuge, at 2500**xg**, for 5 minutes. The supernatant was transferred to a microcentrifuge tube (1.5ml

capacity) and neutralised using 5M potassium carbonate. The pH was monitored using pH indicator solution (Merck, pH4-10). The perchlorate precipitated as the K^+ salt and carbon dioxide was evolved. The tube was sealed and centrifuged at 12000xg for 2-3mins using a high speed centrifuge (Haemicrofuge, Wifug Ltd, Bradford, U.K.). After centrifugation, the supernatant was transferred to another microcentrifuge tube and stored at -20^{0}C for subsequent metabolite assay.

Papillary muscle.

Papillary muscles were removed from liquid nitrogen and transferred to a glass micro-homogeniser (1ml capacity, Wheaton Scientific, New Jersey, USA.), pre-cooled in frozen CO_2 pellets. A 30μl volume of 6% (w/v) perchloric acid per 10mg sample was added to the tissues and the micro-homogeniser was transferred to an ice-bath to allow slow thawing of the tissue/perchlorate mixture. The tissue and perchlorate were homogenised as thawing occurred and the homogenate was transferred to a microcentrifuge tube for centrifugation. An 8μl volume of 5M potassium carbonate /100μl sample was used for neutralisation of the perchloric acid. The K^+ perchlorate precipitate was removed by high-speed centrifugation, as described previously, and the neutralised extract was stored at -20^{0}C for metabolite determination.

2.3.4 Biochemical determination of enzyme activity and metabolite concentration

Measurements of metabolite concentrations in neutralised tissue extracts and enzyme activities in perfusate samples were performed, using commercially available standard assay methods (Boehringer Mannheim Diagnostics, East Sussex, U.K.), on a Hitachi 705 automatic analyser (Boehringer Mannheim Diagnostics). Most of the methods used are based on the changes in absorbance of NAD(P)H at 340nm where a millimolar extinction co-efficient of 6.22 was used in the calculation of the metabolite concentrations or the rate of enzyme catalysed reactions. All

reagents were prepared according to the manufacturer's instructions. For the analysis of tissue concentrations of adenosine triphosphate and creatine phosphate, samples of the neutralised extract were diluted appropriately in Tris acetate buffer (e.g. 1:100/ 1:250). Measurements of cyclic AMP and lactate were made in undiluted neutralised extract samples.

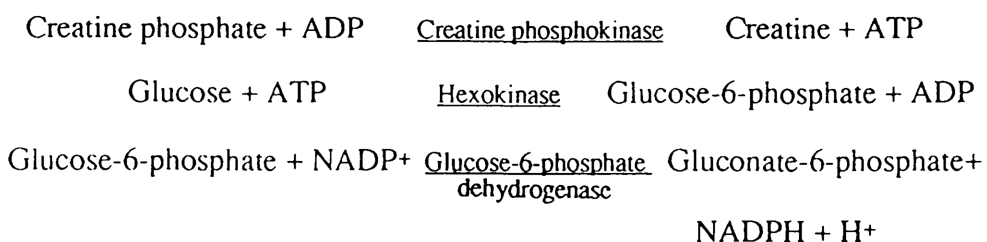
Lactate dehydrogenase

Lactate dehydrogenase (LDH) activity was measured spectrophotometrically (Boehringer Mannheim Kit No. 620092), using a modification of the method described by Weisshaar (1975) in which pyruvate is reduced to lactate and NAD^+:

For the determination of LDH activity, an aliquot of 5µl of perfusate sample was used in an assay volume of 420µl. Final concentrations of the reagents in the assay mixture were 50mM phosphate buffer pH 7.5, 0.18mM NADH and 0.6mM pyruvate. Absorbance was read at 340nm and was proportional to LDH activity in the original sample over the range, 0-1200 IU/litre. Where necessary, the samples were diluted and LDH activity was measured within this range.

Creatine phosphokinase

Creatine phosphokinase (CK) activity was measured spectrophotometrically (Boehringer Mannheim Kit No. 763870), using a modification of the method described by Gruber (1978) in which NADPH is produced in the oxidation of glucose-6-phosphate:



An aliquot of 10µl of perfusate or undiluted neutralised extract sample was used in an assay volume of 420µl. Final concentrations of the reagents in the assay mixture were 0.1M imidazole buffer pH 6.7, 20mM glucose, 10mM magnesium acetate, 2mM EDTA, 2mM ADP, 5mM AMP, 2mM NADP, 20mM N-acetyl-

cysteine, 10μM di-adenosine pentaphosphate, >2.5 IU/ml hexokinase, >1.5 IU/ml glucose-6-phosphate dehydrogenase and 30mM creatine phosphate. Absorbance was read at 340nm and was proportional to CK activity in the original sample over the range, 0-1500 IU/litre. Where necessary, the samples were diluted and CK activity was measured within this range.

Pyruvate dehydrogenase

The frozen tissue powder (50mg) was extracted in 1ml of ice-cold medium comprising 100mM potassium phosphate pH 7.3, 2mM EDTA, 1mM dithiothreitol and 50μl of rat serum/ml. The last two components were added on the day of use. The sample was disrupted in this medium using a Polytron homogeniser (setting 5, 1cm probe, Kinematic GmbH, Lucerne, Switzerland). EDTA was added to chelate Mg^{++} thus preventing any alteration to the existing tissue level of active, non-phosphorylated PDH_a by PDH_a kinase and PDH-phosphatase activity. After homogenisation, the sample was centrifuged (10,000xg for 1 min) and an aliquot of the infranatant removed for measurement of PDH_a content.

Pyruvate dehydrogenase (PDH) activity was measured using the method described by McCormack and Denton (1989). This assay utilises the acetylation of p-(p-aminophenylazo) benzenesulphonic acid (AABS) in the determination of PDH activity:

Pyruvate + NAD^+ + CoA $\xrightarrow{\text{Pyruvate dehydrogenase}}$ acetyl CoA + CO_2 + NADH

Acetyl CoA + AABS $\xrightarrow{\text{Arylamine acetyltransferase}}$ acetylated AABS + CoA

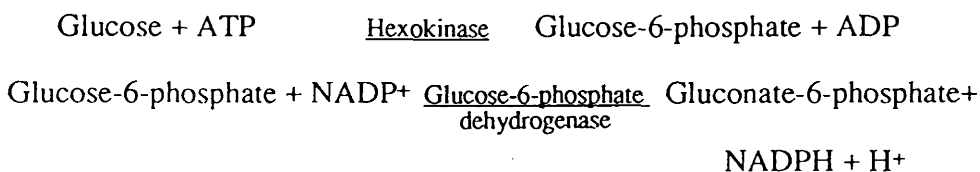
To 1.5ml of buffer (100mM Tris, 0.5mM EDTA and 1mM $MgSO_4$ pH 7.8 containing 20μl/ml of AABS solution and 0.2μl/ml of mercaptoethanol added on the day of the assay), 20μl of a substrate mixture, containing 36mg thiamin pyrophosphate, 23mg NAD^+, 9mg pyruvate and 7.5mg CoA dissolved in 1ml H_2O, was added. The decrease in optical density was followed at 460nm after the addition of 20μl arylamine acetyltransferase and 10-200μl of the extracted sample. AABS was prepared as the sodium or Tris salt in water at 1mg/ml. The millimolar

extinction co-efficient of AABS was 6.5 at this wavelength. AABS was obtained from Pfaltz and Bauer, Stamford, CT0690; arylamine acetyltransferase was kindly supplied by Dr.J.G. McCormack, then of Dept.Biochemistry, University of Leeds.

The amount of PDH present in the active form (PDH_a), was expressed as a percentage of the total amount of PDH (PDH_t) present in the sample. This was assayed, as described above, after the conversion of all of the PDH-phosphate in a portion of the sample to PDH_a. This was achieved by incubation with 20-40μl of pig heart PDH-phosphate phosphatase, in the presence of 25mM $MgCl_2$ and 1mM $CaCl_2$, at 30^oC, for 15 minutes. PDH-phosphate phosphatase was also kindly supplied by Dr. McCormack.

Glucose

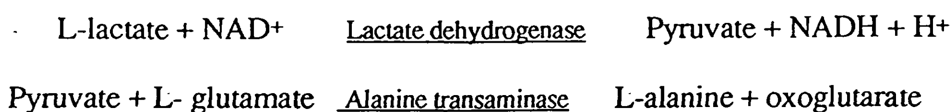
Glucose concentration was measured spectrophotometrically (Boehringer Mannheim Kit No. 704067), using a modification of the method described by Schmidt (1961) in which NADPH is produced in the oxidation of glucose-6-phosphate:



An aliquot of 3μl of perfusate or undiluted neutralised extract sample was used in an assay volume of 400μl. Final concentrations of the reagents in the assay mixture were 69.5mM phosphate buffer pH 7.7, 3.97mM Mg^{++}, 1.29mM ATP, 1.29mM NADP, >1.1 IU/ml of hexokinase and >1.9 IU/ml of glucose-6-phosphate dehydrogenase. Absorbance was read at 340nm and was proportional to the glucose concentration in the original sample over the range, 0-50mM. Where necessary, the samples were diluted and glucose concentration was measured within this range.

Lactate

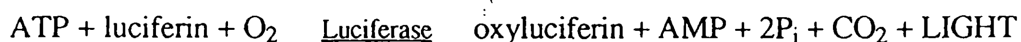
Lactate concentration was measured spectrophotometrically (Boehringer Mannheim assay kit, No.256773), using a modification of the assay method described by Noll (1974) in which lactic acid is oxidised to pyruvate and NADH:



An aliquot of 5µl of perfusate or neutralised extract sample was used in an assay volume of 400µl. Final concentrations of the reagents in the assay mixture were 4mM NAD, 0.43M carbonate buffer pH 10, 54mM L-glutamate, >1.9 IU/ml of alanine transaminase (glutamine-pyruvate aminotransferase), >31 IU/ml of lactate dehydrogenase and 16mM sodium chloride. Absorbance was read at 340nm and was proportional to the lactate concentration in the original sample over the range, 0-11.1mM. Where necessary, the samples were diluted and the lactate concentration was measured within this range.

Adenosine triphosphate

Adenosine triphosphate (ATP) was determined using a bioluminescent assay (ATP monitoring kit, No.1243-102, BioOrbit, Turku, Finland.), based on the quantitative measurement of light produced by the reaction:



An aliquot of 100µl of diluted neutralised extract was added to 400µl 0.1M Tris acetate/2mM EDTA buffer pH 7.75. Standard ATP solutions were also prepared in Tris acetate buffer to give final concentrations of 50-500 pmols per assay tube. The total volume (500µl) was kept constant for each assay sample. The reaction was started by adding 100µl of ATP monitoring reagent which contained Firefly luciferase, D-luciferin, 50mg bovine serum albumin, 0.5mmoles magnesium acetate and 0.1µmoles inorganic phosphate reconstituted in 10mls of distilled water.

The concentration of ATP in the tissue extract sample was compared to the standard ATP sample using a luminometer (Model No. 1250, LKB Wallac, Turku, Finland.). A range of ATP concentrations over which luminescence was directly

proportional to concentration was established using standard solutions and the ATP content of tissue extracts was estimated within this range. Where necessary, samples were diluted within this range.

Creatine phosphate

Quantitation of creatine phosphate (CrP) depends upon the complete conversion of creatine phosphate to ATP in the presence of Mg^{++}, adenosine diphosphate (ADP) and creatine phosphokinase (CK). The ATP produced in this reaction was measured using the method described in the previous section. Standard CrP solutions were prepared in Tris acetate buffer to give final concentrations of 50 - 500 pmols per assay tube. The efficency of the conversion of CrP to ATP was determined by comparison to standard solutions of ATP which were assayed over the same concentration range and under identical conditions.

For the determination of CrP-derived ATP, 100μl of diluted neutralised extract was added to an assay mixture containing 340μl of 0.1M Tris acetate/2mM EDTA buffer pH 7.75, 50μl of 0.1mM ADP, 5μl of 0.3M Mg^{++} acetate and 5μl of CK (1mg protein/ml). The total assay volume (500μl) was kept constant for each sample. The assay tubes were incubated for 30 minutes in a water bath maintained at $25^{0}C$. At the end of this period, 100μl of ATP monitoring reagent was added, and luminescence measured, as described in the previous section.

The ATP measured after this conversion represented both ATP and CrP-derived ATP. The amount of CrP in the sample was calculated by subtracting the ATP concentration measured, using the method described in the previous section from the amount determined after this conversion step.

Glycogen

For the measurement of glycogen, around 500mg of powdered, freeze-clamped heart tissue was transferred to round bottom tubes pre-cooled in frozen CO_2 pellets. After the addition of 1ml of 30% (w/v) potassium hydroxide, the tubes were capped and heated in a boiling water bath for 30 minutes. At the end of this period, the tubes were removed from the water bath, cooled and 1ml of ethanol was added.

The tubes were reheated until the boiling point of the sample was reached and precipitation of glycogen had occurred. The tubes were then removed from the water bath, allowed to cool at room temperature and centrifuged at 2500xg for 10mins using a bench centrifuge. The supernatant was discarded and the tubes were inverted for 30 minutes to allow excess potassium hydroxide to drain from the glycogen pellet. After the addition of 1ml of 0.6M hydrochloric acid, the glycogen was hydrolysed in a boiling water bath for 2 hours. The glucose concentration was measured using the method described previously (see *Glucose*) and glycogen levels were expressed in glucose equivalent units.

Cyclic AMP

Cyclic AMP (cAMP) was measured using an assay system ([^{3}H] cAMP assay kit, No.TRK 432, Amersham International plc.), based on competition between unlabelled cAMP and a fixed quantity of [^{3}H] cAMP for binding to a protein which has a high specificity and affinity for cAMP. The amount of radio-labelled protein-cAMP complex formed is inversely related to the amount of unlabelled cAMP present in the assay sample. Measurement of the protein-bound radioactivity enables the amount of unlabelled cAMP in the sample to be calculated. The protein bound cAMP is separated from the unbound cAMP by adsorption of the free nucleotide onto coated charcoal which is removed by centrifugation. An aliquot of the supernatant is removed and [^{3}H] cAMP measured by scintillation counting.

The assay mixture contained 50μl of undiluted neutralised sample extract, 50μl of [^{3}H] cAMP and 100μl of binding protein. In addition, a zero blank sample comprising 50μl of buffer, 50μl of [^{3}H] cAMP and 100μl of binding protein, was included in the assay. Standard cAMP solutions were prepared to give final concentrations of 1-16 pmols per assay tube. The total assay volume of each sample (200μl) was constant. The assay tubes were vortexed and incubated at 4^{0}C for two hours in an ice-bath. After this period, 100μl of charcoal reagent was added to each tube and the samples were centrifuged for 2-3 mins at 12000xg. Aliquots of 200μl were removed to vials containing 4mls scintillation fluid

(Optiphase, Beckman Instruments (UK) Ltd, High Wycombe, Buckinghamshire, U.K.) and radioactivity (cpm) was counted for a period of 3 minutes using a scintillation counter (LS 5000CE, Beckmann Instruments (UK) Ltd.). A range of cAMP concentrations over which cpm was directly proportional to concentration was established using standard solutions and the cAMP content of tissue extracts was estimated within this range.

Protein assay

The assay procedure used was as described in the commercially available Peirce BCA protein assay kit (Pierce 23225X, USA). Protein determinations were made using the 2 hour incubation at room temperature protocol. Standard bovine serum albumin concentrations ranging from 0.2 - 2mg/ml produced a linear plot from which the concentrations of the unknown protein samples were measured.

Long chain acylcarnitines

Long-chain acylcarnitines (C>10) are insoluble in PCA and were measured as carnitine after alkaline hydrolysis of the extracted protein pellet (Pearson *et al.*, 1974).

Heart tissue was extracted in 6% (w/v) perchloric acid (PCA), as described in Section 2.3.3. The extracted protein pellet was re-suspended in 1ml of 0.5M KOH and the sample was incubated in a water-bath for 60 minutes at 65^{0}C. During the incubation, the pellet was homogenised, using a PTFE rod, to ensure maximum hydrolysis of the acylcarnitine. After the incubation, the sample was centrifuged at 2500xg and the alkaline supernatant was transferred to another tube. The sample was cooled on ice and acidified by the addition of 40µl of 70% (w/v) PCA. At this stage, a white precipitate formed and the sample was re-centrifuged. The supernatant was transferred to another tube and the pH was adjusted to pH 7 using 6M KOH. The pH was monitored using pH indicator solution (Merck, pH 4-10). A 50µl aliquot of the neutralised sample was assayed for carnitine content.

Standard concentrations of carnitine and long-chain acylcarnitine, over the range 2-20 nmoles, were assayed simultaneously and the concentrations of the unknown

samples were determined with reference to the standards.

Carnitine assay

The assay method used was a modification of that described by Bohmer *et al.* (1974) and is based on the production of acetylcarnitine in the following reaction:

A 50µl aliquot of sample or carnitine standard solution was added to an assay mixture containing 50µl of 0.2mM acetyl-CoA, 50µl of 10mM 5,5'-dithiobis-(2-nitrobenzoic acid), 50µl of [^{14}C] acetyl-CoA (equivalent to 18.5µBq) and 50µl of 0.05M Tris buffer pH 7.4. The reaction was started by the addition of 50µl of carnitine acetyltransferase (40 IU/ml). The samples were vortexed and incubated in a water-bath for 30 minutes at 37°C. At the end of the incubation period, 200µl of each sample was removed and passed through plastic syringe columns (1cm x 6cm) containing 5g of Dowex 2x[Cl^{-}] (200-400 mesh) suspended in water before use. Radiolabelled acetylcarnitine was eluted from the column using 2 x 500µl volumes of water. A 200µl aliquot of the eluted sample was added to 10mls scintillation fluid (ReadySafe, Beckman Instruments (UK) Ltd.) and radioactivity was counted for 3 minutes (Packard Tri-carb 460, Canberra Packard Ltd, Pangbourne, Berkshire, U.K.).

2.3.5 Conversion of wet tissue weight to dry tissue weight

Frozen powdered tissue was weighed in a tinfoil weighing boat and transferred to a vacuum oven (Fisons Ltd, U.K.) maintained at a vacuum pressure of 800 mbar and heated to 80-100°C for 24 hours. After this time, the samples were allowed to cool to room temperature under vacuum. The samples were then transferred to a portable vacuum dessicator and quickly reweighed to determine the dry weight. A wet/dry weight conversion factor was calculated by dividing the wet weight of the sample by the dry weight and metabolite concentrations were expressed as dry weight values.

2.3.6 Preparation of palmitate solution

In perfused heart experiments, it was necessary to modify the preparation of the perfusate to allow the addition of palmitic acid. In these experiments, albumin (fatty acid content < 0.02%, Cat No A-7030, Sigma.) was dissolved in water and the appropriate physiological salts were added to give a 3% (w/v) albumin solution. Palmitic acid (Na+ salt, Cat No P-9767, Sigma.) was dissolved in hot water (70^{0}C) and added slowly to a second solution of 3% (w/v) albumin dissolved in water. The appropriate physiological salts were added to give a 1.5mM palmitate : 3% albumin solution. These final solutions were vacuum-filtered through cellulose nitrate filters (0.45μm, Cat No 7184-004, Whatman Ltd, Maidstone, U.K), gassed and heated to 37^{0}C prior to perfusion.

2.3.7 Electrophysiological measurements in the papillary muscle preparation

Electrode preparation

Glass capillary microelectrodes (Clark CG 150F-15) were formed using a microelectrode puller (Sutter Instruments) and filled with 3M potassium chloride solution. The standard tip diameter of the electrode was 3μ and the resistance to a 1nA current was 60megaΩ.

Intracellular recordings

The microelectrode was placed in an electrode holder and lowered onto the surface of the tissue using a micromanipulator. A satisfactory single cell impailment was demonstrated by stable recordings of Vmax and action potentials. The Vmax represents a measure of the fast sodium current (phase 0, see Figure 1.6) and is normally around 150V/sec. The normal action potential duration is around 200msec and the resting diastolic potential is around -85mV. The action potential signal was digitised using an A-D converter (MacLab) and displayed and stored on

an Apple Macintosh computer.

2.3.8 Preparation of heart mitochondria

The isolation of heart mitochondria was carried out essentially as described by McCormack and Denton (1984). Female guinea-pigs were killed by cervical dislocation and the hearts immediately removed. Each heart was transferred to a tube containing 10ml of ice-cold buffer (pH 7.4) containing 250mM sucrose, 20mM Tris-HCl, 2mM EGTA and 1% bovine serum albumin (STEA). The tissue was disrupted using a Polytron (Kinematica) homogeniser (setting 5, 2x2sec bursts).

Differential centrifugation was used to isolate the mitochondria. The homogenates were centrifuged at 1000**xg** for 90 seconds at 4^{0}C in a refrigerated superspeed centrifuge (Sorvall RC-5B). The resultant supernatant was centrifuged at 10,000xg for 10 minutes at 4^{0}C. The pellet produced was resuspended in 2-3ml of ice-cold buffer without albumin (STE) and re-centrifuged at 10,000xg for 10 minutes at 4^{0}C. The pellet was carefully resuspended in 1ml of STE buffer. The metabolic viabilty of the mitochondria was assessed using a Clark oxygen electrode. Coupling ratios ($\pm$ ADP) greater than 3-6 were routinely obtained using 2-oxyglutarate, malate or succinate as substrates.

This chapter describes the standard procedures applied to the experiments performed in this thesis. Any modification to these methods are specified in the relevant chapters

CHAPTER THREE

CHARACTERISATION OF ISCHAEMIC AND HYPOXIC MODELS IN THE PERFUSED GUINEA-PIG HEART PREPARATION

3.1 INTRODUCTION

The development of models for the study of ischaemia in the mammalian heart has recently been reviewed (Opie, 1992). In general, two different isolated heart preparations have been used under *in vitro* conditions. The earliest of these, the retrogradely perfused heart, was first described by Langendorff (1895). More recently, the working heart was developed by Neely *et al.* (1973). In the Langendorff preparation, coronary perfusion is maintained by the aortic pressure, and although the heart does not pump in the physiological sense, this model has been widely employed in the study of contractile and metabolic changes to cardiac function due to ischaemia. In the working heart preparation, perfusion is in the normal direction and physiological "work" is induced by pumping against an imposed afterload.

Under *in vitro* conditions, ischaemia has been achieved in these models either by subjecting the whole heart to ischaemia insult by reducing or stopping flow, or by producing a localised area of ischaemia by occlusion of one or more major coronary arteries. The latter method has been criticised on the basis that accurate definition of the ischaemic area is difficult and therefore the presence of normal tissue in the ischaemic biopsy cannot be excluded (Neely *et al.*, 1973).

Several different conditions of oxygen and substrate deprivation have been used to delineate the effects of ischaemia. *Anoxia* defined as the total absence of oxygen but with continued perfusion of the coronary vasculature, *hypoxia* in which oxygen supply is reduced and perfusion is again maintained, and *low* or *no-flow ischaemia* where the oxygen and substrate supply are both limited by a reduction or complete arrest of perfusion; removal of metabolic products is similarly diminished. Low and no-flow ischaemic conditions are most representative of the clinical situation although it is uncertain which of these conditions predominates during ischaemia in man (Hearse, 1980).

In this chapter a Langendorff-perfused isolated guinea-pig heart method was

used to investigate the functional and metabolic changes induced by both hypoxia and ischaemia *in vitro*. Comparison of these results led to the choice and development of a global low-flow ischaemia model which was used in further studies of the effects of ischaemia described in the subsequent chapters.

3.2 RESULTS AND DISCUSSION

3.2.1 The perfused guinea-pig heart preparation

The isolated guinea-pig heart preparation, described in section 2.3.1, was used in all of the experiments described in this chapter. The aim of these experiments was to assess the changes to contractile function and metabolic characteristics induced in the heart by hypoxia, no-flow ischaemia and low-flow ischaemia. These studies also examined the effects of electrical pacing on the response of the heart to these experimental conditions. Each heart was perfused for a 30 minute equilibration period during which electrical stimulation, when used, was introduced and transducer tension was applied. After this time, hypoxic or ischaemic conditions were introduced for a further 30 minutes. In control hearts, normoxic perfusion was continued during this period.

3.2.1.1 Contractile function and the metabolic characteristics of the perfused heart at the end of the equilibration period

The contractile and metabolic stability of the control hearts during 30 minutes of normoxic perfusion was assessed by a comparison with hearts perfused for the 30 minute equilibration period only. In unpaced hearts (n=6), contractile force at the end of the equilibration period was 1.6 ± 0.3g. During the control perfusion period, contractile force was relatively stable and after 30 minutes was 107.8 ±

2.9% of the measurement made at the end of equilibration. Diastolic tension was 4.7 ± 0.4g after equilibration and decreased to 84.8 ± 2.4% of this value during the control perfusion period. The spontaneous heart rate which was 260 ± 5 beats/min at the end of the equilibration period did not change significantly during the control perfusion period.

At the end of the equilibration period in paced hearts (n=8), the contractile force and diastolic tension were 0.9 ± 0.08g and 4.6 ± 0.2g, respectively. During the control perfusion period, these parameters decreased to 86.9 ± 3.9 and 81.2 ± 5.8% of control, respectively. The electrical pacing rate was 323 ± 10 beats/min.

The metabolic status of the unpaced and paced normoxic control hearts are shown in Tables 3.1 and 3.2, respectively. In comparison to the values obtained from perfused hearts at the end of the equilibration period, the tissue lactate concentrations in the normoxic groups were significantly greater ($p < 0.01$) than that measured at the end of equilibration (unpaced 2.3 ± 0.7 vs 8.1 ± 0.8μmol/gm dry wt.; paced 4.3 ± 0.3 vs 5.5 ± 1.8μmol/gm dry wt.). However, compared to the changes of tissue lactate in hypoxic and ischaemic hearts, these differences were relatively small. The glycogen content of the paced normoxic hearts was lower ($p < 0.05$) than that measured at the end of equilibration (118.5 ± 20.5 vs 157.6 ± 5.9μmol/gm dry wt.). This could reflect increased energy requirements of paced hearts since there was no change to the glycogen content of unpaced hearts. The concentrations of ATP, CrP and cAMP in the unpaced and paced normoxic hearts did not change during the 30 minute normoxic perfusion period.

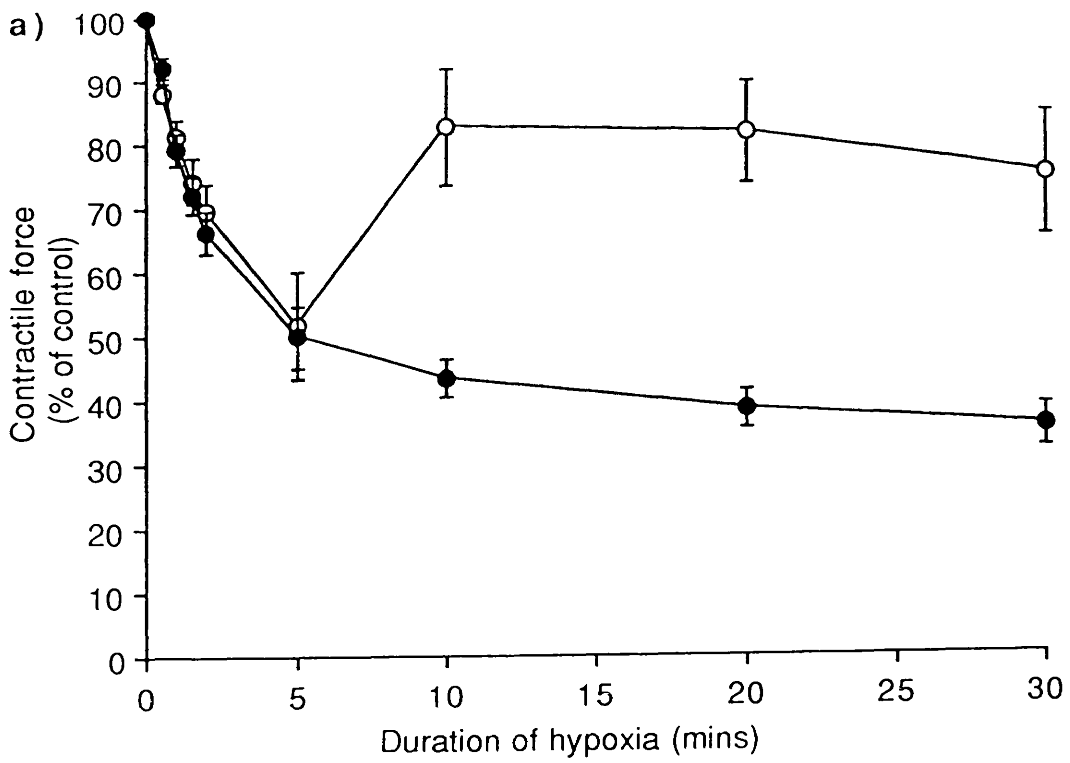
3.2.2 The effects of hypoxia

3.2.2.1. The effects of hypoxia on the contractile function of the unpaced perfused heart.

Control measurements of contractile force prior to hypoxia were 1.0 ± 0.1g.

Figure 3.1 The effects of hypoxia on the contractile function of unpaced and paced perfused guinea-pig hearts

Simultaneous measurements of the changes in a) contractile force and b) diastolic tension during 30 minutes of hypoxia in unpaced (open circles, n=4) and paced hearts (closed circles, n=4). Prior to hypoxia, the hearts were perfused for 30 minutes with physiological salt solution (containing 11.7mM glucose and gassed with 95% oxygen:5% carbon dioxide, $PO_2 = 450$ - 650mmHg) and a stable contractile response was obtained. Electrical pacing was set at 25% above the spontaneous rate as described in section 2.3.1. Hypoxia was induced by gassing the physiological salt solution with 95% nitrogen : 5% carbon dioxide (pO_2~35mmHg). The values shown are mean ± S.E. expressed as % of pre-hypoxic values.


a)
Contractile force
(% of control)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Duration of hypoxia (mins)

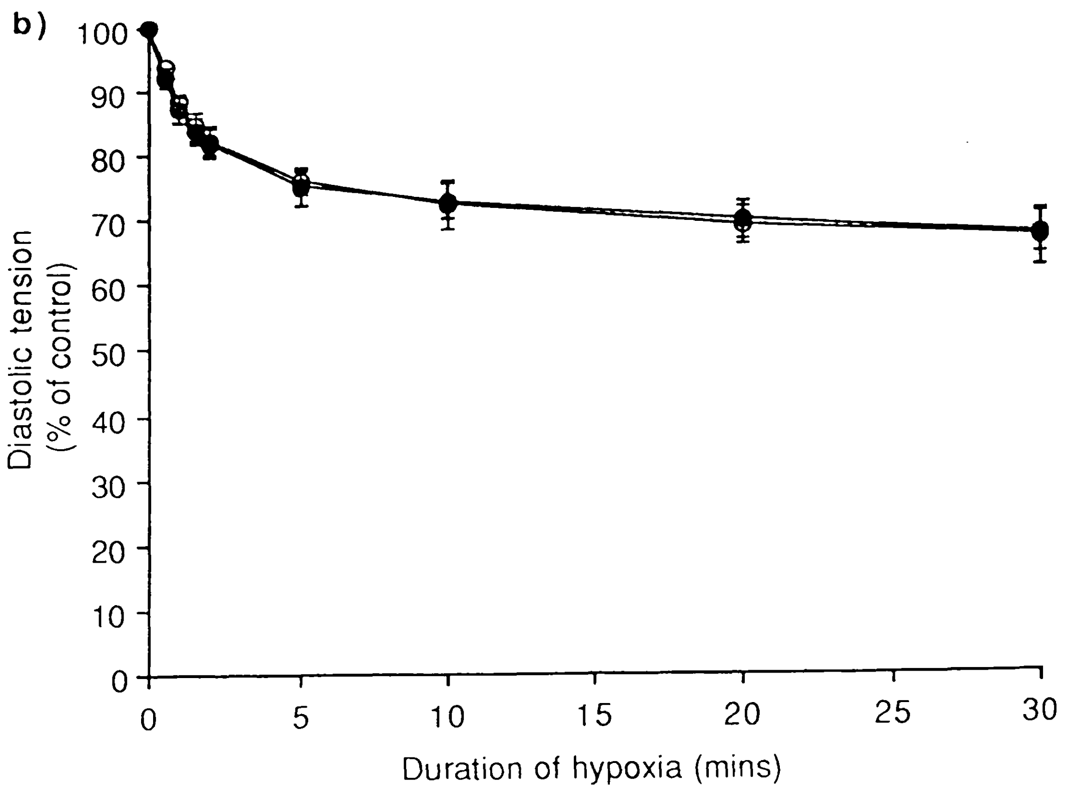
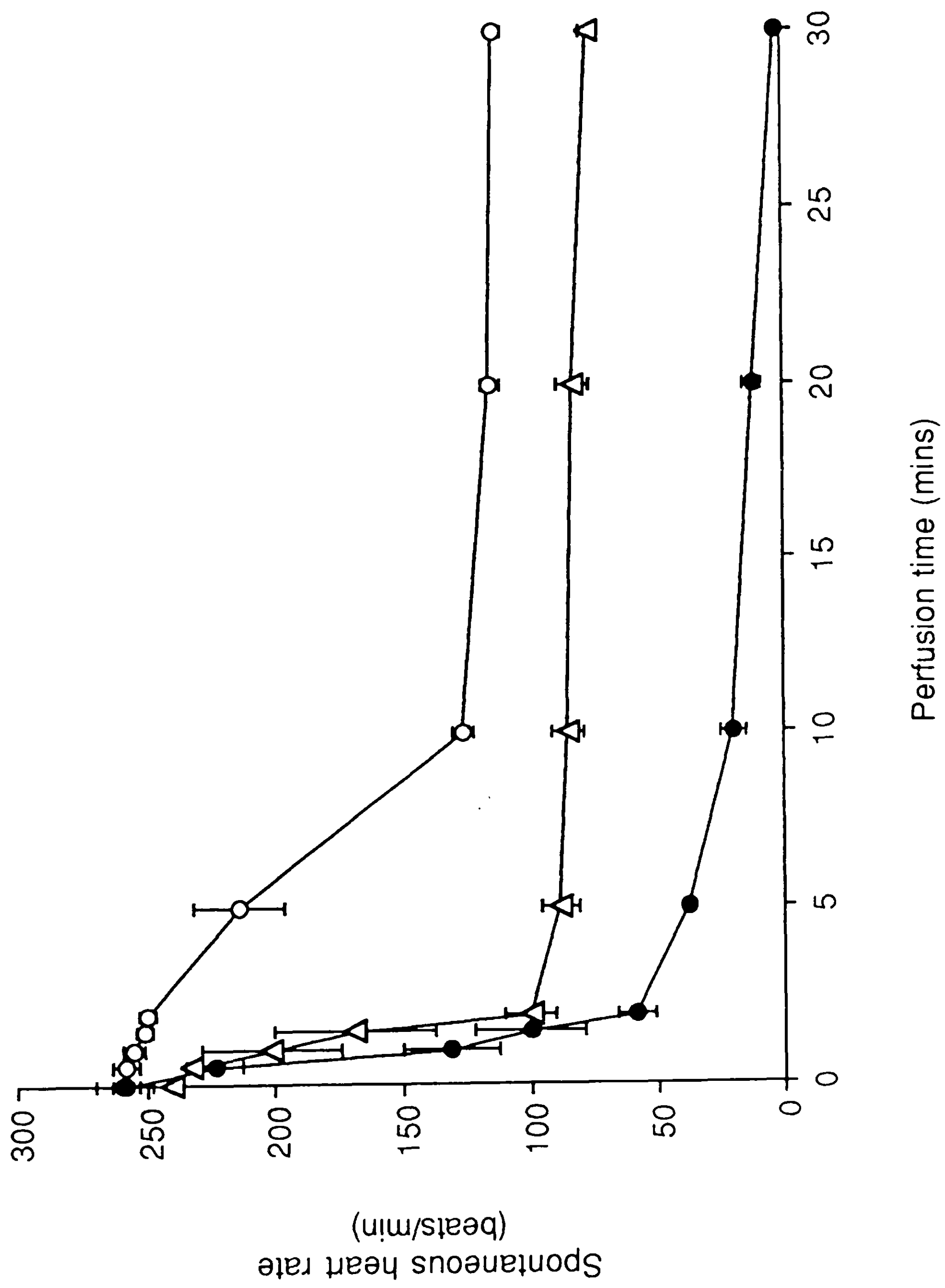

b)
Diastolic tension
(% of control)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Duration of hypoxia (mins)

Figure 3.2 The comparative effects of hypoxia, no-flow and low-flow ischaemia on the spontaneous beating rate in unpaced perfused guinea-pig hearts

Unpaced hearts were perfused under hypoxic (open circles, n=5), no-flow ischaemic (closed circles, n=7) and low-flow ischaemic (open triangles, n=4), conditions for 30 minutes as described in section 2.3.1. Control spontaneous beating rates were recorded prior to the introduction of hypoxia or ischaemia. The values shown are mean ± S.E. expressed as % of the control spontaneous beating rates.


Spontaneous heart rate
(beats/min)
300
250
200
150
100
50
0
0
5
10
15
20
25
30
Perfusion time (mins)

The effects of hypoxia on contractile force was biphasic and after an initial decline to 51.7 ± 8.5% of control, contractility recovered to 82.6 ± 5.1% of control after 10 minutes of hypoxia (Figure 3.1a). At the end of the hypoxic period, contractile force was 74.6 ± 9.7% of control. The observed recovery of contractility could have been related to slowing of the spontaneous beating rate during the hypoxic period (Figure 3.2). because the amplitude of the contractile response was inversely related to the frequency of the heart rate in this preparation.

Diastolic tension decreased during hypoxia in the unpaced hearts. Control diastolic tension was 4.5 ± 0.1g prior to hypoxia. This decreased to 66.6 ± 4.4% of control during the hypoxic period (Figure 3.1b).

The spontaneous beating rate prior to hypoxia was 258 ± 5 beats/min. After 10 minutes of hypoxia, the spontaneous heart rate was approximately 50% of control and decreased to 43.8 ± 1.3 % of control after 30 minutes (Figure 3.2). The onset of this negative chronotropic effect occurred on average at 275 ± 32 seconds after the introduction of hypoxia and was observed in all unpaced hearts.

3.2.2.2. The effects of hypoxia on the metabolic characteristics of the unpaced perfused heart

The metabolic responses of unpaced hearts to hypoxia are compared with normoxia in Table 3.1. At the end of the hypoxic period, the ATP content was virtually unchanged while CrP was reduced to approximately 38% of the normoxic value. The glycogen content was reduced to approximately 73% of the normoxic value after 30 minutes of hypoxia. This showed that even in the presence of glucose, glycogen was utilised during the hypoxic period.

In hypoxic hearts, the tissue lactate concentration was increased to 21.7 ± 1.0μmol/gm dry wt. This showed that despite wash-out by continuous perfusion, lactate accumulated in the tissue. The average rate of lactate efflux was 27.2 ± 0.9μmol/min per g dry wt. Cyclic AMP concentrations were similar in both

normoxic and hypoxic hearts.

3.2.2.3. The effects of hypoxia on the contractile function of the paced perfused heart

In this group, control measurements of contractile force prior to hypoxia were 0.9 ± 0.1g (n=4) and the pacing rate was 300 ± 18 beats/min (25% over the spontaneous rate: see section 2.3.1). After 5 minutes of hypoxia, contractile force had decreased to 49.9± 4.8% of control which was a similar effect observed in unpaced hearts at this time point. However, in the paced hearts, contractile force continued to decrease to 43.3 ± 2.9% at 10 minutes and 35.4 ± 3.2% of control after 30 minutes of hypoxia (Figure 3.1a). In contrast to the response to hypoxia in unpaced hearts, no recovery of contractile force was observed during hypoxia under paced conditions supporting the theory that in unpaced hypoxic hearts, contractile force may be affected by changes in heart rate.

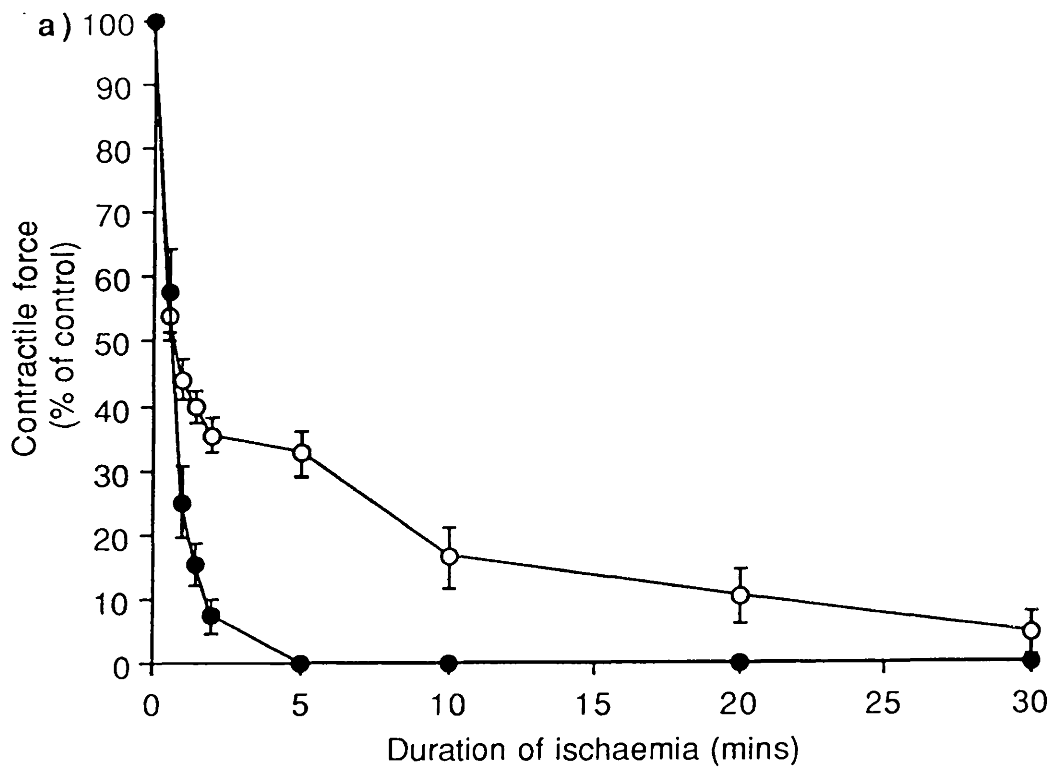
The reduction of diastolic tension in paced hearts was very similar to the unpaced measurements. Control diastolic tension was 4.8 ± 0.2g prior to hypoxia and decreased to 67.4 ± 3.1% after 30 minutes of hypoxia (Figure 3.1b).

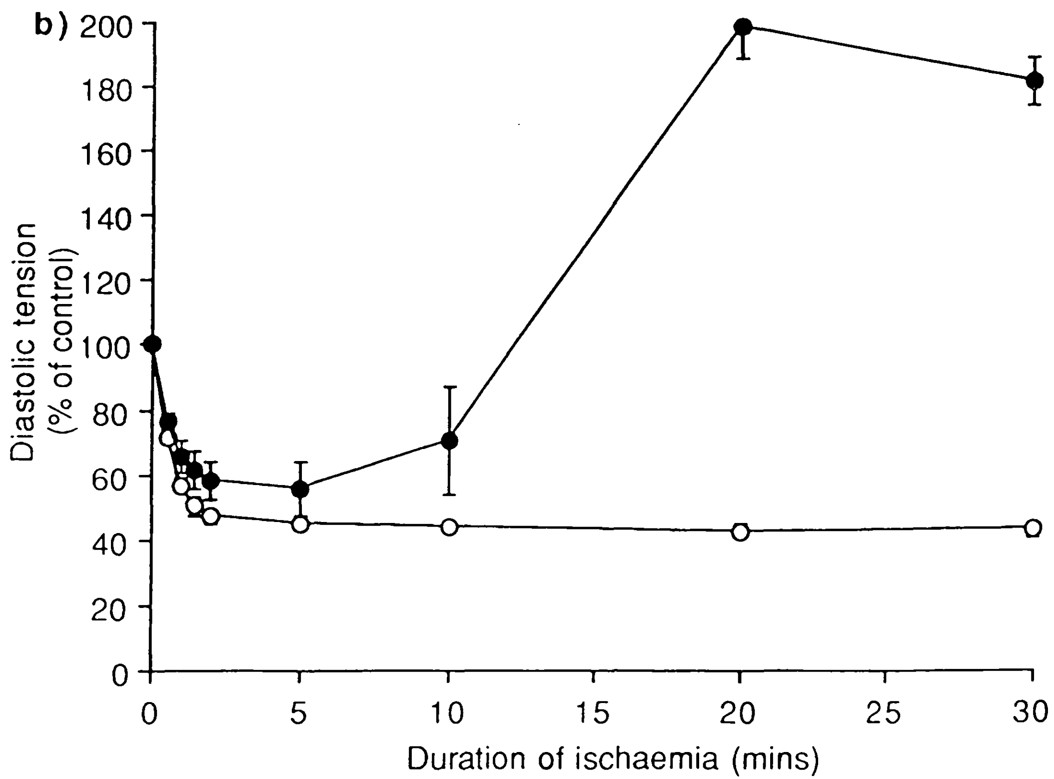
3.2.2.4. The effects of hypoxia on the metabolic characteristics of the paced perfused heart

The metabolic responses of paced hearts to hypoxia are compared with normoxia in Table 3.2. During hypoxia in paced hearts, ATP, CrP and glycogen content were all reduced to approximately 50% of the normoxic values which was greater than the reduction observed in the unpaced hypoxic hearts. This result could be explained by the prevention of compensatory slowing of the heart rate in paced hearts and may account for the inability of these hearts to maintain contractile force during hypoxia. The tissue lactate content of paced hypoxic hearts was 3-

Figure 3.3 The effects of no-flow ischaemia on the contractile function of unpaced and paced perfused guinea-pig hearts

Simultaneous measurements of the changes in a) contractile force and b) diastolic tension during 30 minutes of no-flow ischaemia (NFI) in unpaced (open circles, n=5) and paced (closed circles, n=4) hearts. Prior to NFI, the hearts were perfused with physiological salt solution (containing 11.7mM glucose and gassed with 95% oxygen:5% carbon dioxide, PO_2 = 450 - 650mmHg) and a stable contractile response was obtained. Electrical pacing was set at 25% above the spontaneous rate as described in section 2.3.1 No-flow ischaemic conditions were induced by stopping the perfusion pump. The values shown are mean ± S.E. expressed as % of pre-ischaemic values.


a)
Contractile force
(% of control)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Duration of ischaemia (mins)


b)
Diastolic tension
(% of control)
200
180
160
140
120
100
80
60
40
20
0
0
5
10
15
20
25
30
Duration of ischaemia (mins)

fold greater than in normoxic hearts. These tissue concentrations were similar to those reported for unpaced hearts in Table 3.1. The average rate of lactate efflux was 34.8 ± 3.5μmol/min per g dry wt. This increase in lactate release was slightly higher than in the unpaced hearts. This indicated that although tissue accumulation was similar under paced and unpaced conditions, the overall production of lactate during hypoxia in the paced hearts was higher.

Cyclic AMP concentrations, as in unpaced hearts, were similar in both the normoxic and the hypoxic groups.

3.2.3. The effects of no-flow ischaemia

During ischaemia the myocardium may experience different degrees of perfusion. The following series of experiments were carried out to examine the effects of complete cessation of perfusion on cardiac function and metabolism

3.2.3.1. The effects of no-flow ischaemia on the contractile function of the unpaced perfused heart.

Control measurements of contractile force prior to no-flow ischaemia were 1.2 ± 0.1g. No-flow ischaemia caused a rapid and progressive decrease of contractile force in the unpaced heart. Within 30 seconds, the force of contraction had decreased to 54.1 ± 3.9% of control and was 4.4 ± 3.3 % of control at the end of the 30 minute ischaemic period (Figure 3.3a). This loss of contractile force was more rapid and to a greater degree than observed in unpaced hypoxic hearts and although a reduction in heart rate occurred, there was no recovery of contractility during the ischaemic period (see section 3.2.2.1).

Control diastolic tension prior to no-flow ischaemia was 4.1 ± 0.3g. In comparison to hypoxia (Figure 3.1b), the decrease in diastolic tension was also greater and rapidly fell to around 45% of control after only 2 minutes. This initial

effect was almost maximal and at the end of the ischaemic period, diastolic tension was 42.9 ± 2.2% of control (Figure 3.3b).

The control spontaneous beating rate recorded prior to no-flow ischaemia was 259 ± 11.0 beats/min (Figure 3.2). Ischaemia caused a rapid decrease in heart rate to 50% of control after only 90 seconds. The onset of this negative chronotropic effect was approximately four times faster than observed in hypoxia and occurred on average at 71.3 ± 7.9 seconds after the introduction of ischaemia. As in hypoxia, this reduction in rate was observed in all hearts and at the end of the ischaemic period the spontaneous rate was 2.0 ± 1.5 beats/min. Although this reduction in heart rate did not result in a recovery of contractile force as was observed in unpaced hypoxia, several additional factors may affect contractile activity under no-flow conditions. These factors include, loss of perfusion pressure which would result in a reduction of intraventricular pressure, total loss of oxygen and substrate supply, and accumulation of lactate and other metabolites.

3.2.3.2. The effects of no-flow ischaemia on the metabolic characteristics of the unpaced perfused heart

The changes in the metabolic characteristics of the unpaced heart during no-flow ischaemia are shown in Table 3.2.

After 30 minutes of no-flow ischaemia, the ATP content was unchanged but creatine phosphate was markedly reduced to approximately 30% of the normoxic values. The glycogen concentration was not decreased. In addition to the reduced energy requirements of these hearts, the accumulation of lactate which is known to inhibit glycolysis, may also have contributed to the lack of glycogen utilisation.

Tissue lactate concentrations in the no-flow ischaemic hearts were increased 10-fold from normoxic concentrations and were much higher than those observed in the unpaced hypoxic hearts (see Table 3.1). Cyclic AMP concentrations at the end of the unpaced no-flow ischaemic period were slightly but significantly higher than

the normoxic concentration

3.2.3.3. The effects of no-flow ischaemia on the contractile function of the paced perfused heart

In the paced hearts, control measurements of contractile force prior to no-flow ischaemia were 0.7 ± 0.1g and the pacing rate was 302 ± 9 beats/min. Within only 30 seconds, the force of contraction had decreased to 57.8 ± 6.5% of control (Figure 3.3a). After 2 minutes, contractile force was 7.3 ± 2.6% of control compared to the corresponding measurement of 35.4 ± 2.8% of control in unpaced hearts. In the paced heart, contractility had completely stopped after 5 minutes of no-flow ischaemia, whereas under unpaced conditions, contractile force was not reduced to this level even after 30 minutes.

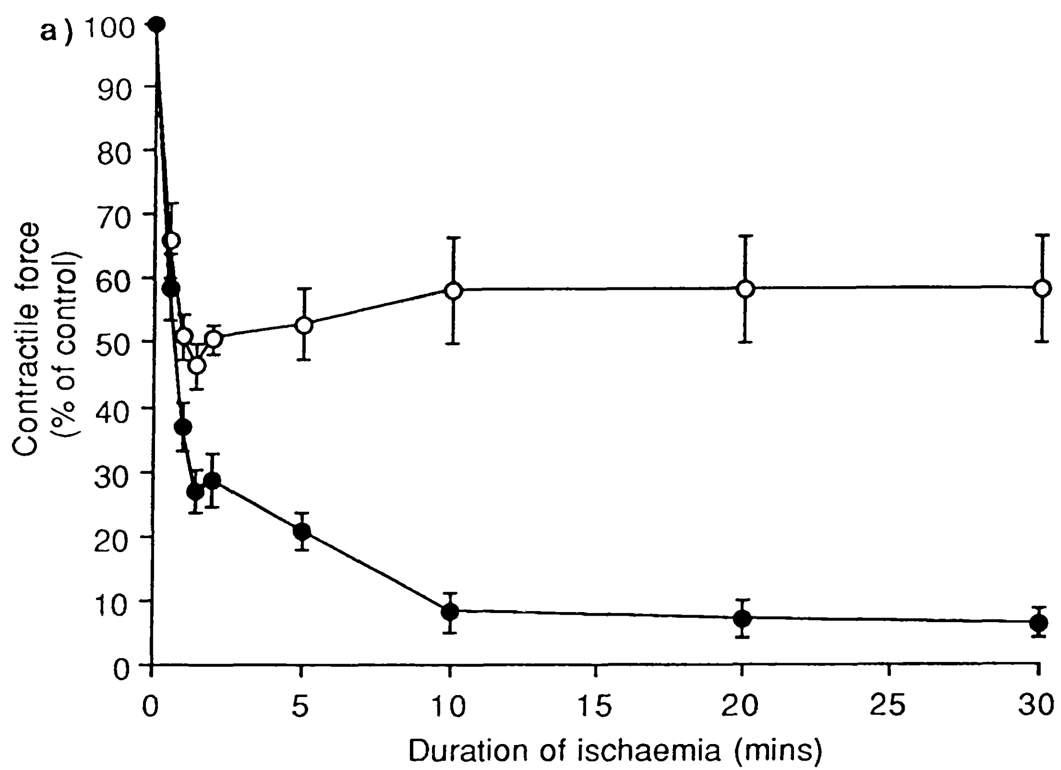
Control diastolic tension prior to no-flow ischaemia was 4.6 ± 0.3g. After 5 minutes of no-flow ischaemia, diastolic tension had decreased to 55.3 ± 8.3% of control compared to 44.6 ± 1.8% of control in unpaced hearts (Figure 3.3b). However, in contrast to the unpaced group, diastolic tension in the paced hearts then increased and was 181.3 ± 7.5% of control after 30 minutes of no-flow ischaemia. This increase in diastolic tension was clearly the result of complete ischaemia coupled with the inability to reduce the spontaneous rate since this effect was not seen in either unpaced no-flow (Figure 3.3b) or paced hypoxic hearts (see Figure 3.1b).

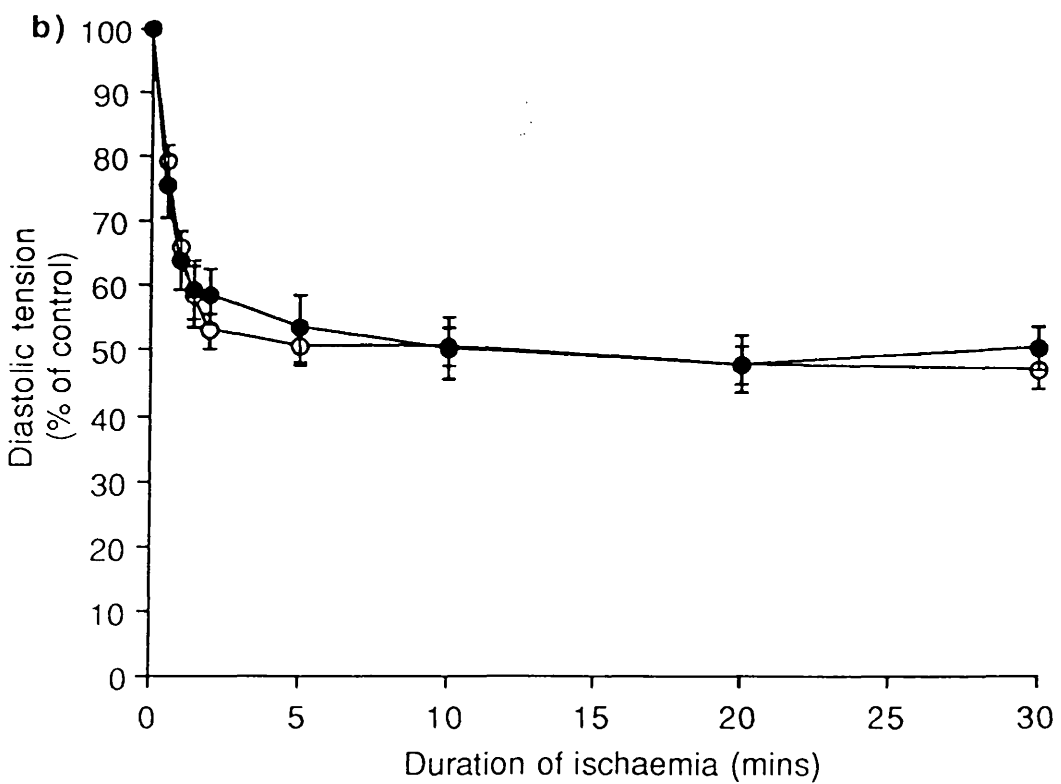
3.2.3.4. The effects of no-flow ischaemia on the metabolic characteristics of the paced perfused heart

At the end of the no-flow ischaemic period, both the ATP and CrP contents were reduced to 30% and 10% of normoxic values, respectively (Table 3.2). There was no evidence of increased glycogenolysis since the glycogen contents of the no-

Figure 3.4 The effects of low-flow ischaemia on the contractile function of unpaced and paced perfused guinea-pig hearts

Simultaneous measurements of the changes in a) contractile force and b) diastolic tension during 30 minutes of low-flow ischaemia (LFI) in unpaced (open circles, n=4) and paced (closed circles, n=4) hearts. Prior to LFI, the hearts were perfused with physiological salt solution (containing 11.7mM glucose and gassed with 95% oxygen : 5% carbon dioxide, PO_2 = 450 - 650mmHg) and a stable response was obtained. Electrical pacing was set at 25% above the spontaneous rate as described in section 2.3.1. LFI conditions were induced by reducing the perfusion rate from a control flow rate of around 9ml/min to 0.7ml/min. The values shown are mean ± S.E. expressed as % of pre-ischaemic values.


a)
Contractile force
(% of control)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Duration of ischaemia (mins)


b)
Diastolic tension
(% of control)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Duration of ischaemia (mins)

flow ischaemic hearts and normoxic hearts were similar. In severe ischaemia, lactate accumulation would inhibit glycolysis and the lactate levels in these hearts were increased 26-fold compared to the normoxic hearts. This was 2.5-fold greater than the increase measured in unpaced no-flow ischaemic hearts (see Table 3.1) and 7-fold greater than the increase observed in paced hypoxia (Table 3.2). Cyclic AMP concentrations in the paced no-flow ischaemic hearts were not statistically significantly different from normoxic values (Table 3.2).

3.2.4 The effects of low-flow ischaemia

In this series of experiments, low-flow ischaemic conditions were induced by reducing the coronary perfusion rate by approximately 90%. The effects of low-flow ischaemia on contractile function and the metabolic status of unpaced and paced hearts were determined.

3.2.4.1. The effects of low-flow ischaemia on the contractile function of the unpaced perfused heart.

The control measurements of contractile force prior to low-flow ischaemia were 1.1 ± 0.1g. As observed in hypoxia and no-flow ischaemia, contractile force decreased during low-flow ischaemia. After an initial reduction to 46.3 ± 3.5% of control after 2 minutes of ischaemia (Figure 3.4a), contractile force stabilised and was maintained at approximately 50-60% of control throughout the remainder of the ischaemic period. At the end of the low-flow ischaemic period, contractile force was 58.1 ± 8.3% of control (Figure 3.4a). Although the spontaneous beating rate slowed in response to low-flow ischaemia, no recovery of contractile force was observed (see section 3.2.2.1).

Control diastolic tension prior to low-flow ischaemia was 4.1 ± 0.3g. After an initial rapid decrease to approximately 60% during the first two minutes of

ischaemia, diastolic tension decreased to 46.9 ± 2.9 % of control at the end of the ischaemic period. No increase in diastolic tension was observed during unpaced low-flow ischaemia (Figure 3.4b).

The spontaneous beating rate prior to low-flow ischaemia was 240 beats/min and low-flow ischaemia caused a 50% reduction in the heart rate within approximately 120 seconds. The onset of this effect was at 75 ± 11.9 seconds after the reduction in flow and was observed in all hearts. The overall reduction was less than in no-flow ischaemia and after 30 minutes of low-flow ischaemia, the spontaneous rate was still approximately 32% of control (see Figure 3.2).

3.2.4.2. The effects of low-flow ischaemia on the metabolic characteristics of the unpaced perfused heart

Low-flow ischaemia did not markedly affect the metabolic status of the unpaced heart. At the end of the 30 minute low-flow ischaemic period, ATP was reduced to 85% and CrP was reduced to 78%. The glycogen content fell to approximately 70% of the normoxic value (Table 3.1). These results were similar to those observed during unpaced hypoxia except for a smaller reduction of CrP in the low-flow ischaemic hearts. However, in comparison to hypoxia, low-flow ischaemia caused a greater reduction in contractile activity which would explain the absence of a marked metabolic difference between these two groups.

Compared to normoxia, tissue lactate increased 3-fold in the low-flow ischaemic group and this was similar to the hypoxic group. The average rate of lactate efflux was 8.8 ± 1.0μmol/min per g dry wt. Cyclic AMP concentrations at the end of the low-flow period in the unpaced hearts were not significantly different from the normoxic concentrations (Table 3.1).

3.2.4.3. The effects of low-flow ischaemia on the contractile function of the paced perfused heart

Control measurements of contractile force prior to low-flow ischaemia in the paced hearts were 0.9 ± 0.1g and the pacing rate in this instance was 309 ± 11 beats/min. Low-flow ischaemia caused a rapid and progressive decrease in contractile force (Figure 3.4a). Within 30 seconds, the contractile force had declined to 58.6 ± 5.1% of control which was similar to the effects observed in unpaced hearts. However, after 30 minutes, contractile force in the paced hearts was only 6.3 ± 2.3% of control compared to 58.1 ± 8.3% of control observed under unpaced conditions.

Control diastolic tension prior to low-flow ischaemia was 4.2 ± 0.5g. Within two minutes of ischaemia the diastolic tension decreased to around 60% of control . After 30 minutes, the diastolic tension was further reduced to 50.1 ± 3.4% of control. No increase in diastolic tension was observed during low-flow ischaemia under paced conditions. This was therefore similar to the effect observed in unpaced low-flow ischaemic hearts (Figure 3.4b) but in contrast to effect observed under no-flow ischaemic conditions (see Figure 3.3b).

3.2.4.4. The effects of low-flow ischaemia on the metabolic characteristics of the paced perfused heart

ATP and CrP were both reduced during paced low-flow ischaemia (Table 3.2). At the end of the ischaemic period, ATP was 58% and CrP was 40% of the normoxic tissue content. This showed that residual flow prevented the substantial depletion of high energy phosphate previously observed under the no-flow conditions (Table 3.2). Glycogen was reduced to approximately 30% of the normoxic value. This was greater than the reduction measured in paced hypoxic hearts (Table 3.2). During hypoxia, glycogenolysis would be expected to proceed

Table 3.1 The effects of hypoxia, no-flow ischaemia and low-flow ischaemia on the metabolic characteristics of unpaced perfused hearts

Unpaced hearts were freeze-clamped after 30 minutes perfusion under normoxic, hypoxic, no-flow ischaemic, or low-flow ischaemic conditions. The conditions associated with the induction of hypoxia and ischaemia and the measurement of high-energy phosphates, glycogen, lactate and cyclic AMP (cAMP) were as described in Chapter 2. The effects of hypoxia and ischaemia were evaluated by a comparison to the normoxic values obtained from hearts perfused with physiological salt solution containing 11.7mM glucose and gassed with 95% oxygen:5% carbon dioxide. The cAMP values are expressed as nmol/gm dry wt. and all other values are μmol/gm dry wt. The values shown are mean ± S.E. Statistical comparison to normoxic control levels was by analysis of variance and application of a 2-sided t-test; *$p<0.05$; ***$p<0.001$.

Experimental Condition	Tissue content				
	ATP	Creatine phosphate	Glycogen	Lactate	cAMP
Normoxia (n=7)	13.1 ± 0.8	14.3 ± 2.4	159.6 ± 8.8	8.1 ± 0.8	2.4 ± 0.2
Hypoxia (n=4)	12.0 ± 0.8	5.5 ± 0.7 ***	115.8 ± 6.9 *	21.7 ± 1.0 *	3.2 ± 1.0
No-flow ischaemia (n=5)	13.2 ± 0.8	4.1 ± 0.4 ***	174.1 ± 18.5	83.5 ± 5.2 ***	4.9 ± 0.7 ***
Low-flow ischaemia (n=4)	11.3 ± 0.6	11.6 ± 0.7	111.6 ± 10.3 *	27.3 ± 3.1 ***	2.8 ± 0.4

Table 3.2 The effects of hypoxia, no-flow ischaemia and low-flow ischaemia on the metabolic characteristics of paced perfused hearts

Paced hearts were freeze-clamped after 30 minutes perfusion under normoxic, hypoxic, no-flow ischaemic, or low-flow ischaemic conditions. Details of the measurements made and the statistical analysis performed are given in the legend for Table 3.1.

Experimental Condition	Tissue content				
	ATP	Creatine phosphate	Glycogen	Lactate	cAMP
Normoxia (n=6)	14.1 ± 0.4	14.9 ± 0.9	118.5 ± 20.5	5.5 ± 1.8	2.1 ± 0.1
Hypoxia (n=4)	7.4 ± 0.3 ***	6.9 ± 0.3 ***	60.5 ± 11.0 **	19.4 ± 1.6 **	1.7 ± 0.1
No-flow ischaemia (n=4)	4.3 ± 1.3 ***	1.2 ± 0.4 ***	102.6 ± 6.3	145.9 ± 7.6 ***	3.3 ± 0.4
Low-flow ischaemia (n=4)	8.2 ± 0.4 ***	6.1 ± 0.9 ***	39.0 ± 5.7 ***	50.8 ± 7.7 ***	3.5 ± 0.2 *

at a higher rate than during low-flow ischaemia. However, unlike hypoxia, low-flow ischaemia would result in a reduction of the exogenous glucose supply which may have caused an increase in glycogen utilisation.

The tissue lactate content of the low-flow ischaemic hearts was increased 10-fold compared to normoxia but remained lower than in the no-flow hearts since lactate was also removed in the perfusate. The average rate of lactate efflux was 12.8 ± 2.4μmol/min per g dry wt. Compared to the normoxic concentration, a small but significant elevation of cAMP was observed in the paced low-flow ischaemic hearts .

3.3 DISCUSSION

These experiments have compared the effects of different degrees of oxygen and substrate debt in the unpaced and paced guinea-pig heart. Many studies have examined the effects of ischaemia and hypoxia in cardiac tissue but the majority have been performed using rat tissue. However, compared to other species, the rat heart shows considerable differences in receptor types (Flynn *et al.*, 1978; Steinfath *et al.*, 1992) and in the excitation-contraction coupling mechanism (Clarke and Patmore, 1984). The guinea-pig heart, which is similar to the human myocardium in these respects, was therefore used in the studies described in this thesis. This thesis would also contribute to the general assessment of the effects of ischaemia in the myocardium since less information is available on such studies in the guinea-pig heart.

Under *in vitro* conditions, the isolated perfused guinea-pig heart proved to be a reliable preparation in that the contractile and metabolic responses in each of the groups investigated were consistent. The ATP content of the normoxic perfused guinea-pig hearts in these studies was similar to the perfused rat heart values reported by van Bilsen et al., (1991) and Lipasti et al., (1984). However, the literature data varies widely depending on the species used and the perfusion conditions applied.

In each group of unpaced hearts, it was clear that changes to the spontaneous heart rate influenced the response of the heart to hypoxia and ischaemia. This was especially so in the case of hypoxia where the continuous supply of exogenous glucose coupled with the removal of metabolites resulted in only minor changes to contractile activity. The effects of hypoxia were more evident in the metabolic response of the heart. The degree of oxidative metabolism was reduced as indicated by the increased concentration of tissue lactate and although ATP levels were not significantly decreased, both creatine phosphate and glycogen levels were reduced. Utilisation of these substrates would maintain ATP at a normal concentration. It was of interest that glycogen levels were reduced even in the presence of glucose. This suggested that even 11.7mM glucose was not sufficient to saturate the glycolytic capacity of the hypoxic-perfused heart.

The extent of the compensatory effect of the changes in the spontaneous beating rate during hypoxia were clearly demonstrated when the same experiment was repeated in paced hearts. Under paced conditions, the increased mobilisation of both creatine phosphate and glycogen failed to sustain ATP at the normal level.

Unpaced hearts subjected to no-flow ischaemia also exhibited negative chronotropism but this effect was very rapid and resulted in almost complete cessation of beating during the ischaemic period. This may have been the result of the inhibitory effect of lactate on glycolysis and the results obtained from the no-flow ischaemia experiments would be compatible with this hypothesis. If this was the case then the elevated lactate levels found in these hearts must have been produced from a small degree of glycogen utilisation in the early stages of the ischaemic episode and metabolism of residual glucose within the tissue. The results from these experiments also showed that while glycogen utilisation was restricted, this was not the case with creatine phosphate. ATP levels were high which may have been the result of the rapid reduction in heart rate coupled to markedly reduced contractile force. This theory is supported by the fact that in paced hearts during no-flow ischaemia, both ATP and creatine phosphate were depleted. However, as in

the unpaced hearts, glycogen levels were not significantly lowered yet lactate levels were found to be even higher than in unpaced hearts. Further experiments to determine relative changes in glycogen and lactate at different time-points in this type of experiment would be required to clarify this finding.

A major difference between unpaced and paced hearts during no-flow ischaemia was the development of contracture (increased diastolic tension). This was associated with low ATP levels and a high tissue lactate concentration. Acidosis and the subsequent increase of intracellular $[H^+]$ is known to antagonise intracellular Ca^{++} binding (Katz, 1973; see section 4.4). If Ca^{++} is involved in contracture development when ATP levels are low (Steenbergen *et al.*, 1990), then these results demonstrated that contracture development was not prevented by the high level of lactate present after no-flow ischaemia. It could also be argued from these results that since lactate levels were high in hearts which developed contracture, some causal link between the two events may exist. However, recent evidence supports the theory that ATP depletion is the major factor in contracture development (Bowers *et al.*, 1992).

The differences between unpaced and paced hearts established in the hypoxia and no-flow ischaemia models was also true for low-flow ischaemia. Low-flow ischaemia had an intermediate effect on the reduction of spontaneous heart rate. Interestingly, in both hypoxia and low-flow ischaemia, after an initial rapid decline, the rate stabilised over the remainder of the perfusion period suggesting that a balance between rate, contractile force and energy supply had been achieved. This was reflected in the maintenance of contractile force at a stable albeit lower level in these hearts. In contrast to hypoxia, no depletion of creatine phosphate was found during unpaced low-flow ischaemia although glycogen had been utilised. During unpaced hypoxia, contractile force increased and the spontaneous rate was maintained at a relatively high level. Contractile activity was therefore greater during unpaced hypoxia than during unpaced low-flow ischaemia. It was therefore possible that the energy requirements of the unpaced low-flow ischaemic heart were

adequately met by glycogen utilisation and residual exogenous glucose supply. The metabolic response to low-flow ischaemia changed under paced conditions. Creatine phosphate and glycogen decreased. Although tissue lactate levels were increased, they were not as high as those measured after no-flow ischaemia. This was presumably due to the removal of lactate by the residual perfusion and would have allowed some degree of glycolysis to continue. This would explain the high level of glycogen depletion compared to no-flow ischaemia. Contracture development was not observed during low-flow ischaemia but the ATP level in low-flow ischaemic hearts was not as low as in no-flow ischaemia.

In conclusion, the ability of the myocardium to exhibit negative chronotropism had an important influence on the contractile and metabolic effects in experimental models of hypoxia and ischaemia. The comparative study of hypoxia, no-flow and low-flow in paced hearts has shown that the changes in myocardial function varied according to the model used (NFI > LFI > Hypoxia) and that only no-flow ischaemia was associated with contracture development. The next chapter examined the potential effects of impaired glucose metabolism in the perfused heart by investigating the effects of glucose-free perfusion on the contractile and metabolic responses to hypoxia and ischaemia.

CHAPTER FOUR

STUDIES ON GLUCOSE AND FATTY ACID METABOLISM DURING NORMOXIA, HYPOXIA AND ISCHAEMIA IN THE PERFUSED GUINEA-PIG HEART PREPARATION

4.1 INTRODUCTION

The previous chapter has described the effects of hypoxia and different grades of ischaemia on the contractile and metabolic response of the isolated perfused guinea-pig heart. The low-flow ischaemia model has been identified as a suitable preparation for further investigations into metabolic factors which may alter the effects of ischaemia on myocardial function. In this chapter, the influence of the two major substrates, glucose and fatty acid, on the metabolic and contractile responses of the heart to low-flow ischaemia have been examined. A protective role for glucose in the ischaemic myocardium has been proposed (Opie and de Leiris, 1979; Opie and Bricknell, 1979) and ischaemia-induced changes to lipid metabolism have been associated with exacerbation of myocardial cell damage (Katz and Messineo, 1981). Since the relative plasma levels of these two substrates *in vivo* have been shown to control their uptake into the myocardium (Randle, 1963), it is important that aspects of both glucose and fatty acid metabolism are addressed.

The effects of glucose-free perfusion in hypoxic and low-flow ischaemic hearts were investigated to demonstrate the potential effects of impaired glucose metabolism and the effects of palmitic acid, a long-chain fatty acid ($CH_3(CH_2)_{14}COOH$) which gives rise to a major accumulation of biologically active metabolites during ischaemia (Idell-Wenger et al., 1978), were studied.

4.2 RESULTS AND DISCUSSION

The contribution of exogenous glucose metabolism to the contractile activity and the metabolic status of the normoxic-perfused heart was studied by perfusing the hearts in the presence and absence of glucose (section 4.2.1). A high incidence of fibrillation was unexpectedly observed during glucose-free perfusion and this was further investigated both in the guinea-pig perfused heart and papillary muscle preparation (section 4.2.2). The glucose-free studies in hypoxia and ischaemia (section 4.2.3) extended the information obtained in the previous chapter by demonstrating the contribution of exogenous glucose to the contractile and metabolic characteristics of the perfused guinea-pig heart . The effects of glucose on lactate production (section 4.2.4) and contracture development during ischaemia (section 4.2.5) were investigated.

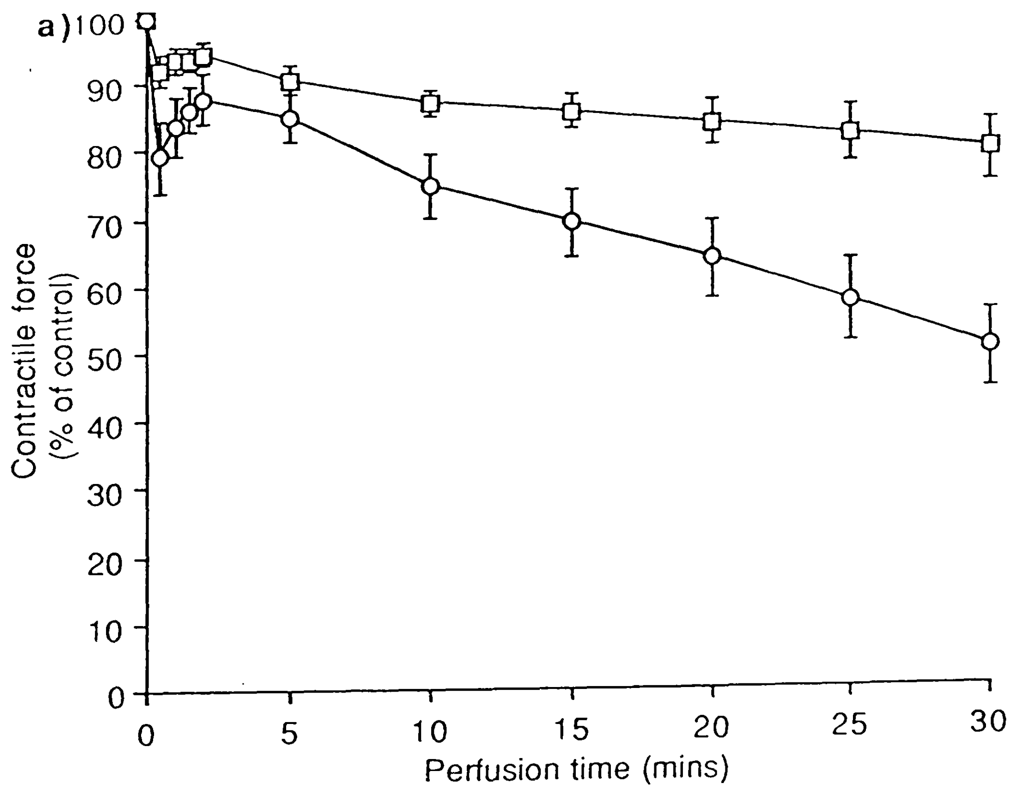
4.2.1. The effects of glucose-free perfusion in the normoxic-perfused heart.

4.2.1.1. The effects of glucose-free perfusion on the contractile function of the normoxic-perfused heart

Glucose-free conditions were produced in the perfusate by substituting glucose with equimolar mannitol. The osmolality of the glucose and mannitol solutions were similar (284 and 287mosmol/Kg, respectively). The contractile effects of glucose-free perfusion, for 30 and 90 minutes, in the paced normoxic heart are shown in Figure 4.1. All contractile changes were expressed as a % of the measurement made at the end of the 30 minute equilibration period prior to the start of glucose-free perfusion.

Figure 4.1 The effects of glucose-free perfusion on contractile force and diastolic tension in the paced normoxic perfused guinea-pig heart

Time-course of changes to contractile force (open circles) and diastolic tension (open squares) during a) 30 minutes (n=3) and b) 90 minutes (n=3) of glucose-free normoxic perfusion. Mannitol (11.7mM) was substituted for glucose and the hearts were paced and perfused as described in Chapter 2. Values are mean ± S.E. and are expressed as % of the contractile values measured prior to the introduction of glucose-free conditions.


a)
100
90
80
70
60
50
40
30
20
10
0
Contractile force
(% of control)
0
5
10
15
20
25
30
Perfusion time (mins)

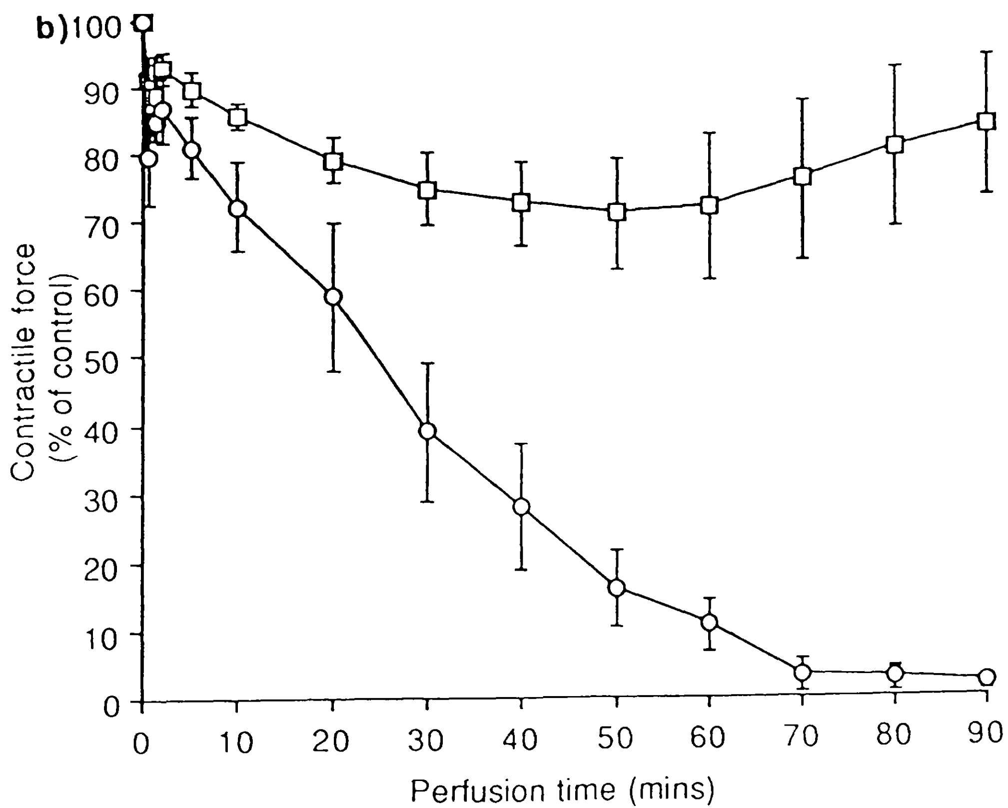
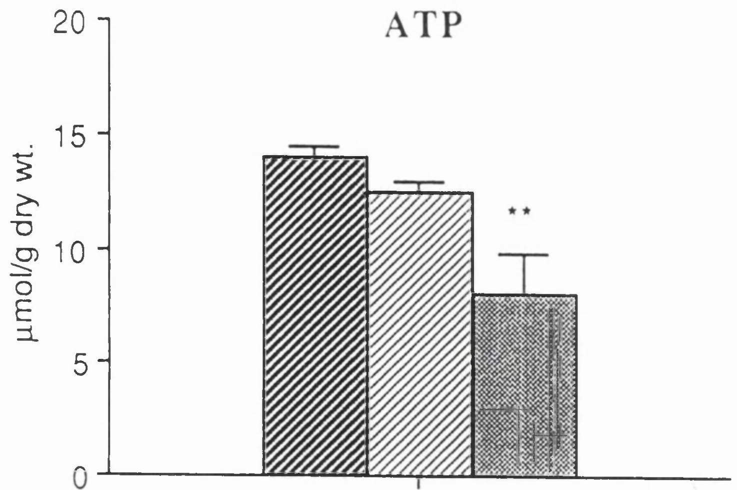
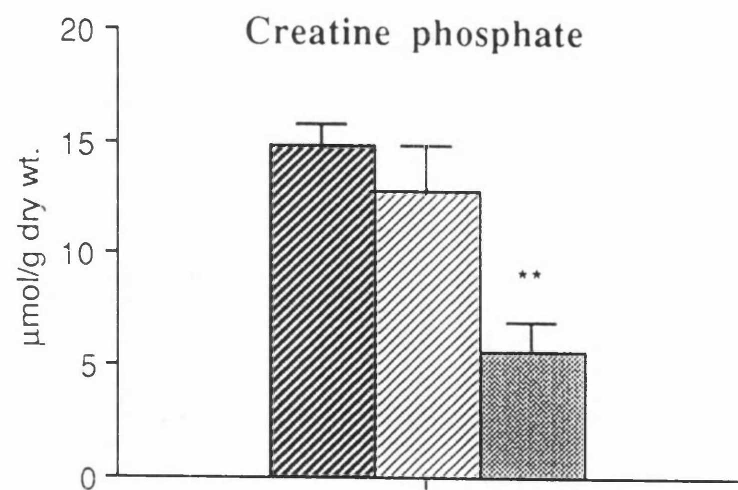

b)
100
90
80
70
60
50
40
30
20
10
0
Contractile force
(% of control)
0
10
20
30
40
50
60
70
80
90
Perfusion time (mins)

Figure 4.2 The metabolic characteristics of the paced guinea-pig heart after glucose-free normoxic perfusion

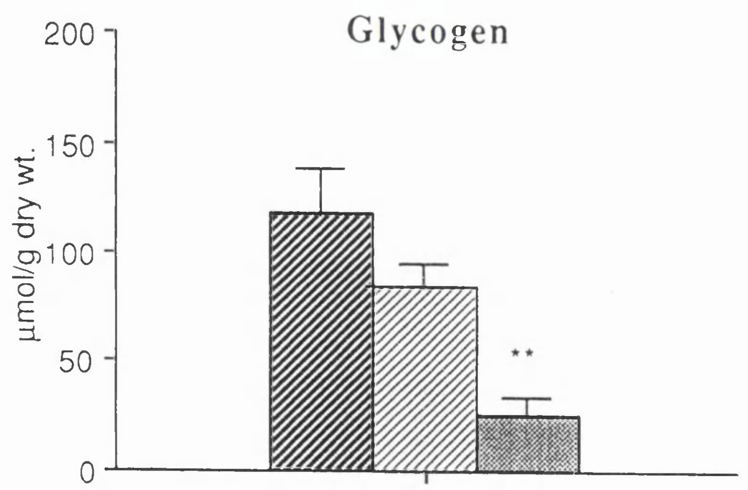
The metabolic status of paced normoxic hearts perfused for 30 minutes under glucose-free conditions was determined as described in Chapter 2. The changes in contractile activity in these hearts are described in Figure 4.2. Values are mean ± S.E. (n=3). Statistical evaluation of the comparison between normoxic values taken from Table 3.3 (n=6, dark hatch bars) and glucose-free normoxia for 30 minutes (n=3, light hatch bars) or 90 minutes (n=3, dark shaded bars) was by analysis of variance and application of Dunnett's t-test (**$p< 0.01$).



ATP
μmol/g dry wt.
20
15
10
5
0
**



Creatine phosphate
μmol/g dry wt.
20
15
10
5
0
**



Glycogen
μmol/g dry wt.
200
150
100
50
0
**

The introduction of glucose-free conditions caused an initial transient decrease in contractile force to 79.2 ± 5.4% of control. Contractile force subsequently recovered to 87.8 ± 3.8% of control after approximately 120 seconds. Thereafter, contractile force progressively decreased to 49.5 ± 5.8% of control during the remainder of 30 minute period (Figure 4.1a). Diastolic tension showed a gradual decline to 78.8 ± 4.5% of control during the 30 minute period (Figure 4.1a).

When glucose-free perfusion was prolonged for 90 minutes, there was a progressive decrease in contractile force to around 40% of control after 30 minutes and 1.8 ± 1.1% of control after 90 minutes. Thus, contractile force was almost completely abolished during the 90 minute period (Figure 4.1b). Diastolic tension was only slightly affected by glucose-free conditions and decreased to 83.2 ± 10.4% during the 90 minute period (Figure 4.1b).

4.2.1.2. The effects of glucose-free perfusion on the metabolic characteristics of the normoxic-perfused heart

Normoxic values for ATP, creatine phosphate, glycogen and lactate were obtained from hearts perfused with glucose for 30 minutes (Figure 4.2). These values are also shown in Table 3.2. After 30 minutes of glucose-free perfusion, ATP, creatine phosphate and glycogen levels remained high and were not significantly different from the normoxic values in glucose-perfused hearts (Figure 4.2). After 90 minutes of glucose-free perfusion, ATP, creatine phosphate and glycogen were significantly decreased (Figure 4.2).

These results showed that in the absence of exogenous glucose, contractile activity in the normoxic heart was supported by the metabolism of endogenous substrates during the 30 minute period. However, the marked decrease of contractile force shown after 90 minutes of glucose-free perfusion was associated with depletion of these energy reserves.

4.2.2. Studies on the mechanism of fibrillation observed in the normoxic-perfused heart during glucose-free perfusion.

Although no significant effects on the endogenous energy reserves had been observed during 30 minutes of glucose-free perfusion, there was a greater tendency towards fibrillation in hearts perfused without glucose. Fibrillation was not preceeded by arrhythmia and did not occur in every heart. This observation was of interest because it has been suggested that ATP produced from glycolysis may have a special role in the maintenance of membrane integrity and electrical stability (Bricknell and Opie, 1978). The relationship between fibrillation and glucose metabolism was investigated by assessing the biochemical status of these hearts at the time of fibrillation.

4.2.2.1. The high-energy phosphate and glycogen content during fibrillation in the normoxic heart perfused in the absence of glucose

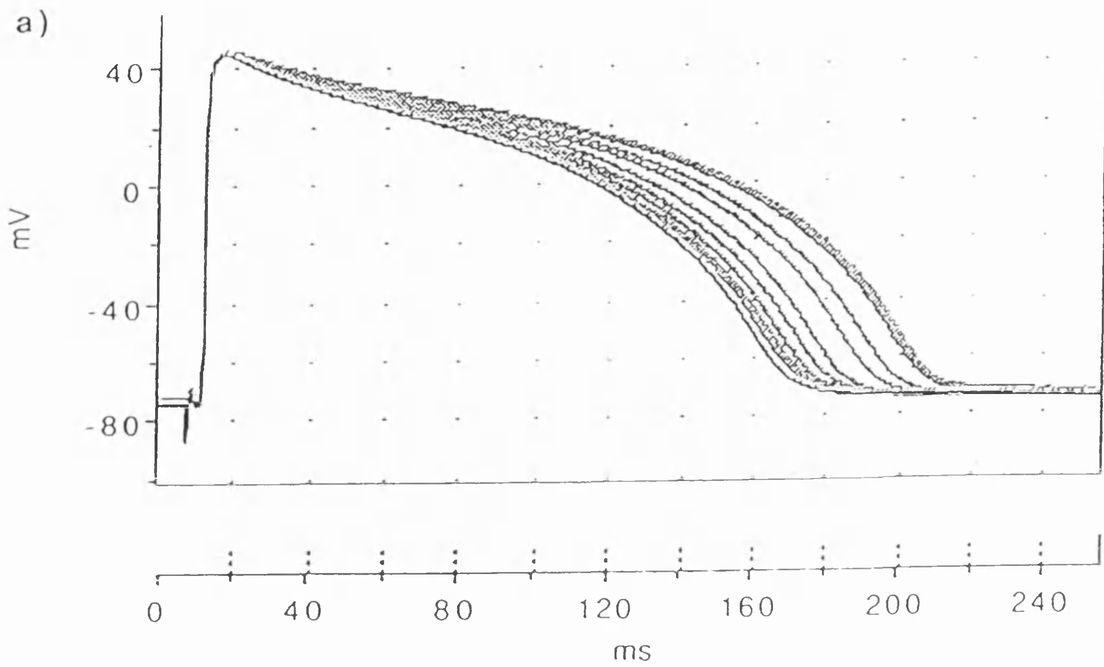
The fibrillation times for 6 hearts were recorded and the metabolic status of each heart, immediately after fibrillation, was determined.

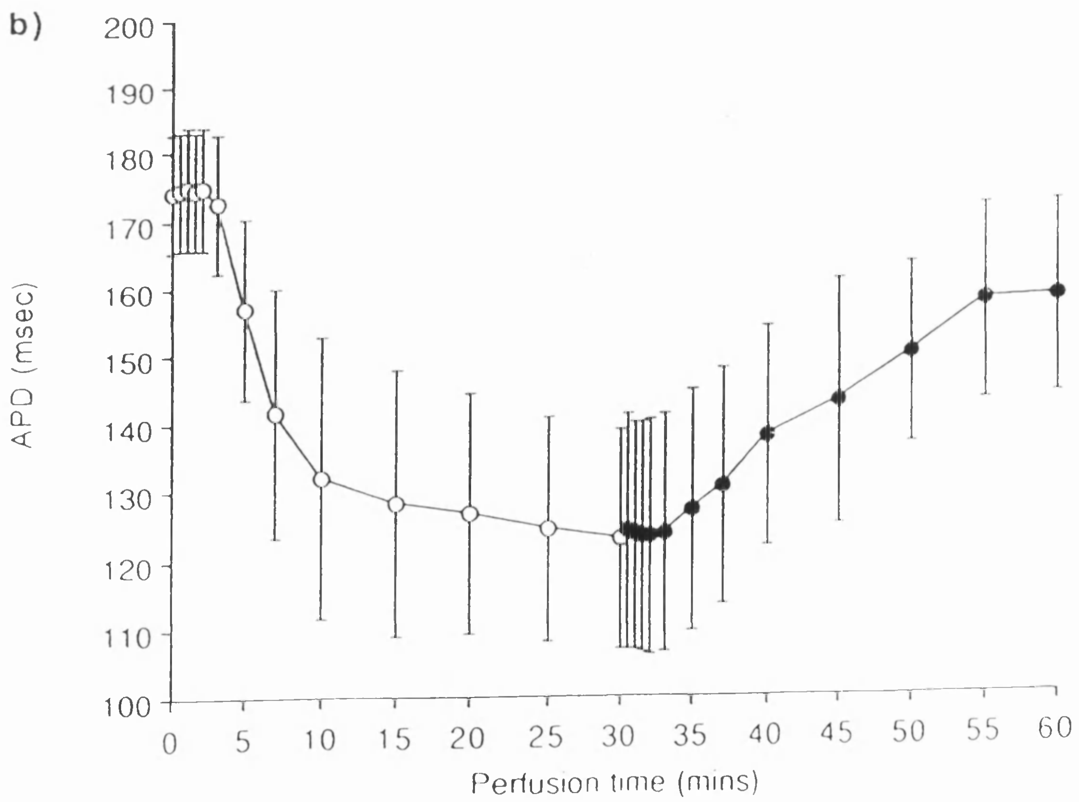
Time to fibrillation	ATP*	Creatine phosphate*	Glycogen*
5 mins	11.4	13.0	81.4
14 mins	11.9	13.1	90.0
30 mins	10.3	8.5	50.7
47mins	10.3	8.2	50.6
28 mins	13.1	13.5	145.1
47 mins	11.5	9.5	76.5

(* all values expressed as µmol/g dry wt.)

Figure 4.3 The effects of glucose-free superfusion on the action potential duration in the normoxic guinea-pig papillary muscle

Figure a) shows a representative trace of the time-dependent decrease in action potential duration (APD) during glucose-free superfusion of the guinea-pig papillary muscle. Recordings were made at 0.5, 1.0, 1.5, 2, 3, 4, 5, 7, 10, 15, 20, 25 and 30 minutes after the introduction of glucose-free conditions. The effects of glucose-free (n=3, open circles) and glucose-free + 10μM glibenclamide (n=3, closed circles) superfusion over a 60 mins normoxic period are shown in Figure b). Maximum depolarisation was measured at the peak of the initial upstroke (around + 40mV) shown in Figure a) and the APD (msecs) shown in Figure b) represents the time to 90% repolarisation from + 40mV. 90% repolarisation is the standard measurement of APD in this type of experiment. Values shown are mean ± S.E.


a)
40
0
-40
-80
mV
0
40
80
120
160
200
240
ms


b)
200
190
180
170
160
150
140
130
120
110
100
APD (msec)
0
5
10
15
20
25
30
35
40
45
50
55
60
Perfusion time (mins)

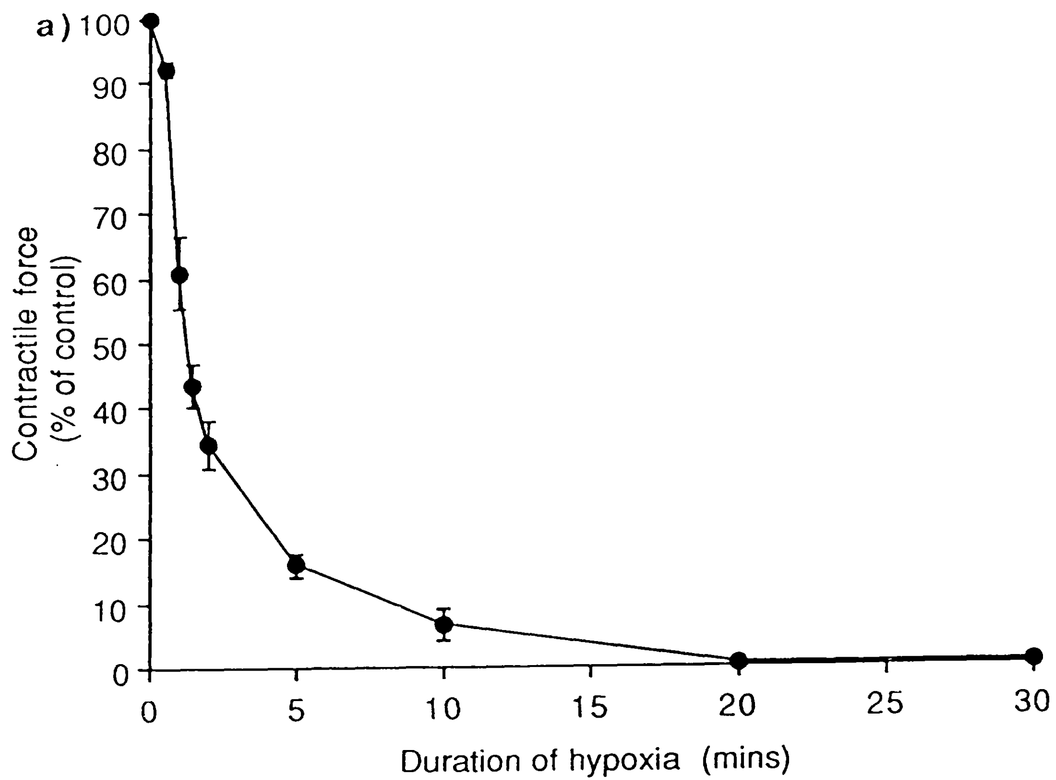
Despite variation between hearts, there was no correlation between the duration of glucose-free perfusion and the measured metabolic characteristics of the hearts which might have explained the incidence of fibrillation. However, it was possible that changes to the stability of the cell membrane may have occurred in the absence of glucose. This was investigated using electrophysiological measurement of the cardiac action potential in guinea-pig papillary muscles which were superfused under similar conditions to those used in the whole heart.

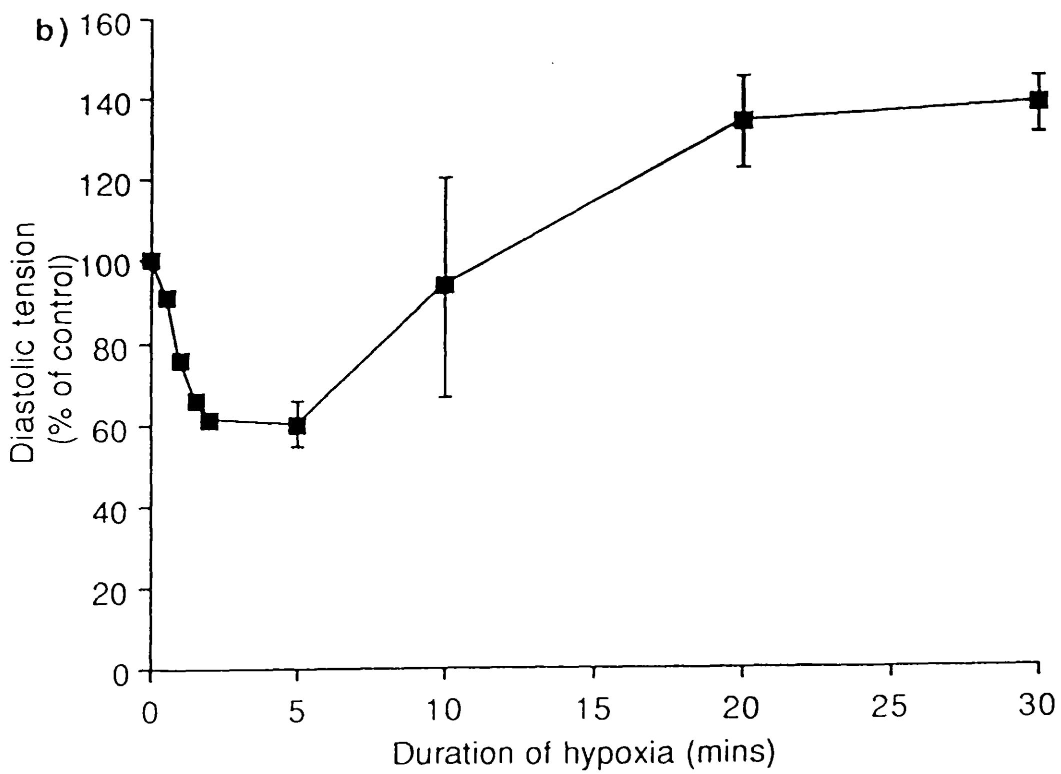
4.2.2.2. Measurement of the cardiac action potential duration (APD) in the isolated guinea-pig papillary muscle during glucose-free superfusion.

The methodology used in these experiments has been described in section 2.3.2. Briefly, normal cardiac action potentials were recorded after a 60 minute equilibration period in bicarbonate-buffered physiological salt solution containing 11.7mM glucose. Glucose-free buffer was introduced and changes in the APD were compared to the normal action potential. A representative recording of the changes to the normal APD in response to glucose-free perfusion is shown in Figure 4.3a. In control experiments, the normal APD measured in the presence of glucose was 177 ± 5msecs (n=9). This value was stable (101 ± 0.3%) over 60 minutes. In the absence of glucose, the APD shortened from 174.1 ± 8.9msec to 122.8 ± 16.0msec ($p < 0.001$) over a 30 minute period. At the end of this period, perfusion for a further 30 minutes with 10μM glibenclamide, still under glucose-free conditions, reversed the shortening of the APD to 157.0 ± 14.0msec ($p < 0.001$ compared to 122.8 ± 16.0msec; Figure 4.3b). Since glibenclamide is a specific blocker of the ATP-dependent K^+ channel (Kantor *et al.*, 1990), this reversal indicated the involvment of these channels in the effect of glucose-free conditions in this preparation. Prolongation of the action potential duration is an effective anti-arrhythmic mechanism (Singh et al., 1980). If the results obtained in the papillary

Figure 4.4 The effects of glucose-free hypoxia on contractile force and diastolic tension in the paced perfused guinea-pig heart

This figure shows the time-course of the changes in a) contractile force and b) diastolic tension during glucose-free hypoxia in paced hearts (n=4). Mannitol (11.7mM) was substituted for glucose and hypoxic conditions were as described for Figure 3.1. Values shown are mean ± S.E. and are expressed as % of the contractile values measured prior to the introduction of hypoxia.


a)
100
90
80
70
60
50
40
30
20
10
0
Contractile force
(% of control)
0
5
10
15
20
25
30
Duration of hypoxia (mins)


b)
160
140
120
100
80
60
40
20
0
Diastolic tension
(% of control)
0
5
10
15
20
25
30
Duration of hypoxia (mins)

muscle experiments are representative of the effect of glucose-free perfusion in the isolated heart , then shortening of the action potential in glucose-free perfused hearts could have precipitated fibrillation through reduction of the refractory period.

4.2.3. The effects of glucose-free perfusion in the paced hypoxic and low-flow ischaemic perfused heart

Hypoxia was used because the high rate of glycolysis associated with hypoxia suggested that this model might be highly sensitive to changes in glycolytic substrate.

4.2.3.1. The effects of glucose-free perfusion on the contractile function of the paced hypoxic-perfused heart.

Control measurements of contractile force prior to glucose-free hypoxia were 0.9 ± 0.1g (n=4) and the pacing rate was 242 ± 10.3 beats/minute. After 5 minutes of paced glucose-free hypoxia, contractile force was reduced to 15.8 ± 1.7% of the control value and after 20 minutes, contractile activity had almost ceased (Figure 4.4a). Thus, the effect of hypoxia on contractile force was greatly increased in the absence of exogenous glucose.

Control diastolic tension prior to glucose-free hypoxia was 4.8 ± 0.3g. This had decreased to 61.4 ± 1.7% of control after 5 minutes of glucose-free hypoxia. Over the remainder of the hypoxic period, diastolic tension increased to 137.1 ± 6.9% of control (Figure 4.4b). This effect was not observed under paced hypoxic conditions in the presence of glucose (section 3.2.2).

4.2.3.2. The effects of glucose-free perfusion on the metabolic characteristics of the paced hypoxic-perfused heart.

The effects of glucose-free hypoxia on the metabolic characteristics of paced hearts after 30 minutes are shown in Table 4.1. ATP and creatine phosphate were reduced to approximately 20% and 25% and glycogen was reduced to 26% of the amount measured in paced glucose-perfused hearts. Tissue lactate concentrations in the glucose-free hypoxic group were increased to approximately 300% of the normoxic value and the average rate of lactate efflux was 3.9 ± 0.4µmol/min per g dry wt. There was no change in the cyclic AMP content.

In hearts subjected to glucose-free hypoxia, high energy phosphates and glycogen were depleted to a greater degree than observed during hypoxia in the presence of glucose (see Table 3.2). Although tissue lactate concentrations were similar in both glucose and glucose-free hypoxic hearts, more lactate was released into the perfusate from hearts provided with glucose (see section 3.2.2.4).

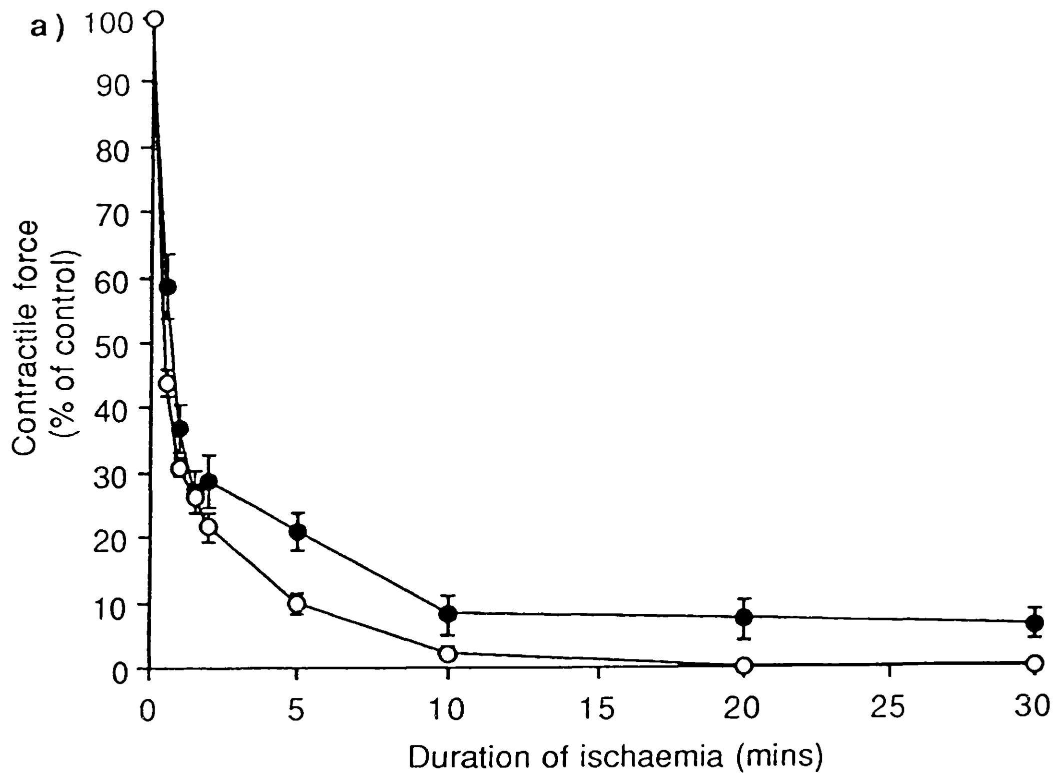
4.2.3.3. The effects of glucose-free perfusion on the contractile function of the paced low-flow ischaemic-perfused heart

Control measurements of contractile force prior to glucose-free low-flow ischaemia were 0.8 ± 0.1g (n=5) and the pacing rate was 324 ± 9 beats/min. Contractile force decreased rapidly after the onset of glucose-free ischaemia and was reduced to 30.9 ± 1.4% of control after 60 seconds. This compared to 36.9 ± 3.6% of control in low-flow ischaemic hearts perfused with glucose (section 3.2.4). After 10 minutes, contractile force had been reduced to 2.1 ± 1.0% of control (Figure 4.5a).

Control diastolic tension prior to glucose-free low-flow ischaemia was 4.5 ± 0.2g. Ischaemic contracture developed within 10 minutes of the start of glucose-

Figure 4.5 Comparison of the effects of low-flow ischaemia with and without glucose on contractile function in the paced perfused guinea-pig heart

This figure compares the effects of low-flow ischaemia on a) contractile force and b) diastolic tension in paced hearts perfused with and without glucose. As in hypoxia (Figure 4.4), contracture development was observed only under glucose-free conditions (n=6, open circles). The data for low-flow ischaemia in the presence of glucose (n=6, closed circles) is taken from Figure 3.4. Values shown are mean ± S.E. and are expressed as % of pre-ischaemic values.


a)
Contractile force
(% of control)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Duration of ischaemia (mins)

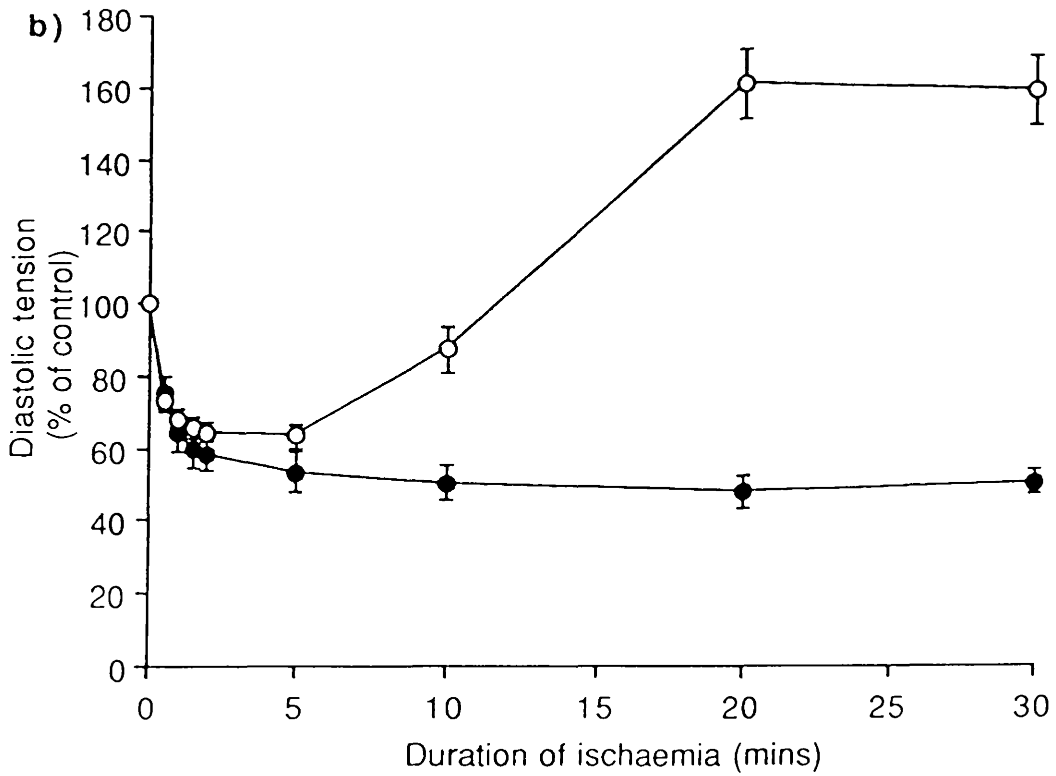

b)
Diastolic tension
(% of control)
180
160
140
120
100
80
60
40
20
0
0
5
10
15
20
25
30
Duration of ischaemia (mins)

Table 4.1 The comparative effects of hypoxia and low-flow ischaemia on the metabolic characteristics of paced guinea-pig hearts perfused in the absence of glucose

At the end of the glucose-free hypoxic and low-flow ischaemic periods (see section 4.2.3), the hearts were freeze-clamped and changes to the normal metabolic characteristics of the tissues were measured as described in Chapter 2. Glucose-free values were compared to the corresponding measurements from normoxic hearts perfused in the presence of glucose (Table 3.2). Statistical evaluation of the differences was made by analysis of variance and application of a 2-sided t-test (**$p<0.01$; ***$p<0.001$). All values are expressed as μmol/g dry wt.

Experimental Condition	Tissue content			
	ATP	Creatine phosphate	Glycogen	Lactate
Normoxia (Table 3.2)	14.1 ± 0.4	14.9 ± 0.9	118.5 ± 20.5	5.5 ± 1.8
Glucose-free hypoxia (n=4)	2.8 ± 0.4 ***	3.8 ± 0.9 ***	31.2 ± 5.9 ***	16.4 ± 3.9 **
Glucose-free low-flow ischaemia (n=6)	3.9 ± 0.6 ***	4.3 ± 0.5 ***	21.9 ± 2.8 ***	10.9 ± 1.5 **

free ischaemia and was 158.3 ± 9.8% of control after 30 minutes (Figure 4.5b). This was in contrast to low-flow ischaemia in the presence of glucose in which no increase in diastolic tension was observed.

4.2.3.4. The effects of glucose-free perfusion on the metabolic characteristics of the paced low-flow ischaemic-perfused heart.

At the end of the glucose-free ischaemic period, ATP and creatine phosphate were both reduced to approximately 28% and glycogen levels were reduced to approximately 18% of the glucose-perfused normoxic value (Table 4.1). Thus, the decrease in high energy phosphate and glycogen was greater under glucose-free conditions than in glucose-perfused low-flow ischaemic hearts (see Table 3.2). These results showed the relative contributions of glucose and glycogen to the cellular energy requirements of the low-flow ischaemic heart.

4.2.4. Lactate production in the paced ischaemic heart

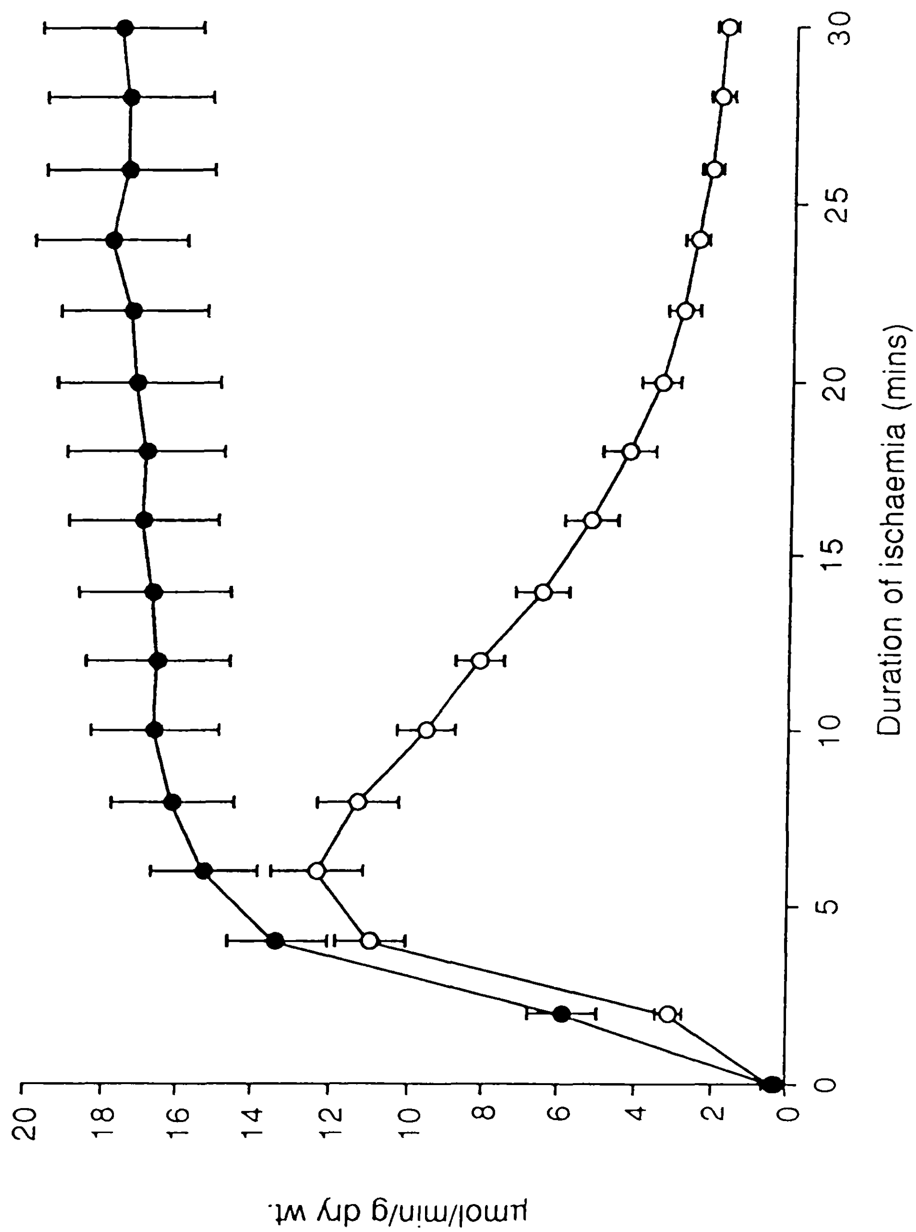
Total lactate production during the low-flow ischaemic period was determined by the combined measurement of lactate efflux collected in overflow perfusate samples and the lactate content of post-ischaemic tissue.

4.2.4.1. Low-flow ischaemia in the presence of glucose

Total lactate efflux during 30 minutes low-flow ischaemia was 480.9 ± 38.9μmol/g dry wt. (n=7). This was equivalent to 16.0 ± 1.3μmol/min per g dry wt. The post-ischaemic tissue content was 46.7 ± 5.0μmol/g dry wt. Therefore the total lactate produced during the ischaemic period was approximately 526μmol/g dry wt.

Figure 4.6 Time-dependent lactate efflux during low-flow ischaemia with and without glucose in the paced perfused guinea-pig heart

The perfusate was collected over 2 minute intervals throughout the 30 minute low-flow ischaemic period and the lactate content of the samples was measured as described in Chapter 2. Lactate efflux during low-flow ischaemia in the presence of glucose (n=5, closed circles) was compared with lactate released during glucose-free low-flow ischaemia (n=6, open circles). Values shown are mean ± S.E.



µmol/min/g dry wt.
0
2
4
6
8
10
12
14
16
18
20
0
5
10
15
20
25
30
Duration of ischaemia (mins)

In order to determine the rate of increase in lactate production during low-flow ischaemia, efflux was measured over 2 minute intervals throughout the 30 minute ischaemic period. There was an initial rapid increase in lactate production from a basal rate of 0.28 ± 0.09μmol/min per g dry wt. to 16.2 ± 1.6μmol/min per g dry wt. after 8 minutes of low-flow perfusion. After this time, lactate was released at a relatively constant rate of 17.2 ± 0.1μmol/min per g dry wt. (Figure 4.6).

4.2.4.2. Low-flow ischaemia in the absence of glucose

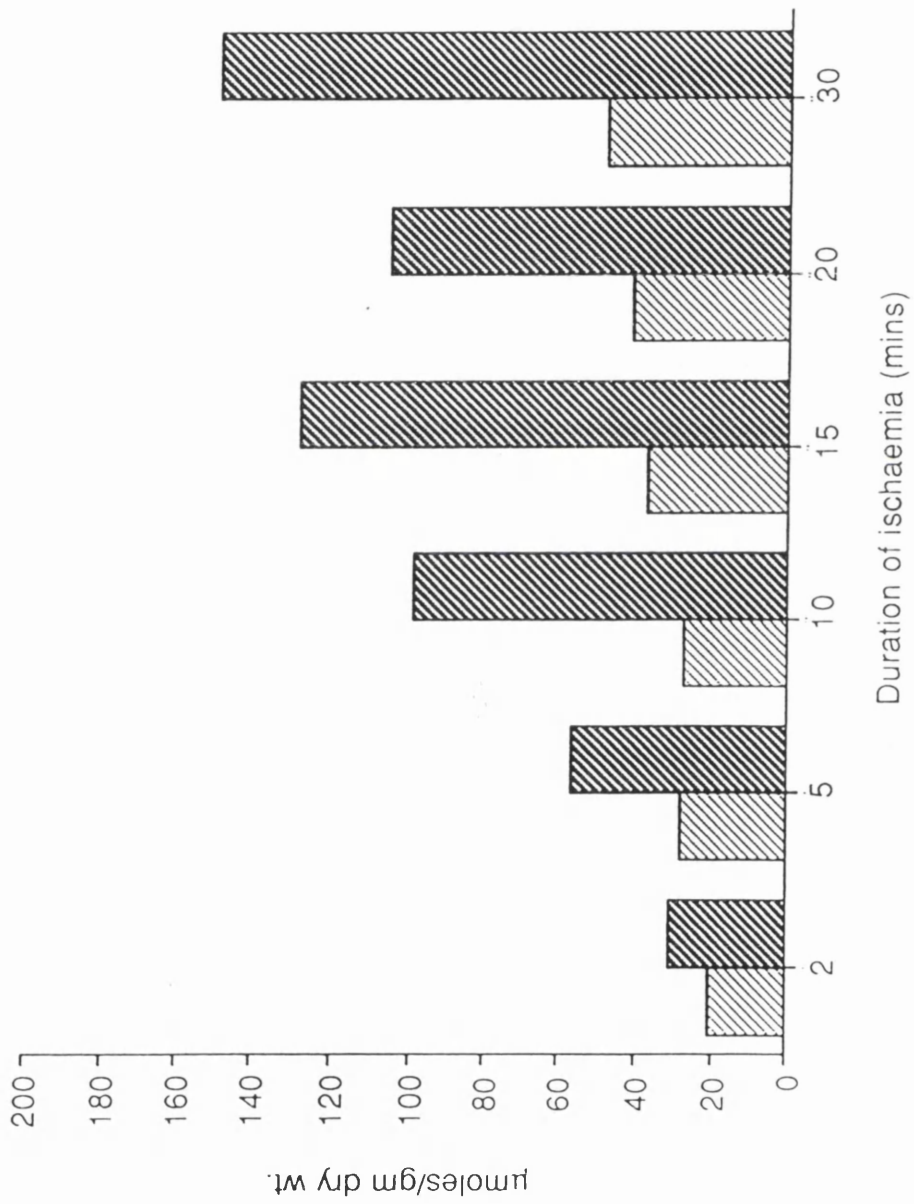
Total lactate efflux during 30 minutes of glucose-free low-flow ischaemia was 87.7 ± 16.9μmol/g dry wt. (n=6). This was equivalent to 2.9 ± 0.6μmol/min per g dry wt. The post-ischaemic tissue content was 12.3 ± 1.7μmol/g dry wt. (n=6). Therefore, the total lactate production under glucose-free conditions, was approximately 100μmol/g dry wt. This was approximately 5-fold less than lactate production during low-flow ischaemia in the presence of glucose. Lactate efflux increased from basal levels of 0.40 ± 0.20μmol/min per g dry wt. to 11.4 ± 0.8μmol/min per g dry wt. during the initial 8 minutes of glucose-free low-flow ischaemia but efflux then decreased progressively to only 1.8 ± 0.2μmol/min per g dry wt. at the end of the ischaemic period (Figure 4.6). Since the post-ischaemic glycogen content of these hearts was very low (Table 4.1), this decrease in lactate efflux could be explained by the loss of glycolytic substrate.

4.2.4.3. No-flow ischaemia

In these hearts, all of the lactate produced during the ischaemic period was retained within the tissue. After 30 minutes of no-flow ischaemia, tissue lactate levels were 153.7 ± 12.0μmol/g dry wt. (n=6). This corresponded to 30% of the total lactate produced during low-flow ischaemia indicating a reduction of

Figure 4.7 Comparison of tissue lactate accumulation during low-flow and no-flow ischaemia in the paced perfused guinea-pig heart

Hearts were freeze-clamped after 2, 5, 10, 15, 20 and 30 minutes of low-flow (light hatch bars) or no-flow ischaemia (dark hatch bars) and tissue lactate levels were measured as described in Chapter 2. Single observations at each time-point were made for the two ischaemic conditions .



μmoles/gm dry wt.
200
180
160
140
120
100
80
60
40
20
0
2
5
10
15
20
30
Duration of ischaemia (mins)

glycolysis in this model.

The rate of lactate accumulation during no-flow ischaemia was measured after 2, 5, 10, 15, 20 and 30 minutes. This enabled a comparison to be made between the rate of lactate production during low-flow ischaemia and no-flow ischaemia (Figure 4.7). The accumulation of tissue lactate was almost linear during the first 15 minutes of no-flow ischaemia but was only slightly increased between 15 and 30 minutes. This showed that glycolysis was inhibited in the later stages of this ischaemic insult.

While lactate production was sustained during low-flow ischaemia in the presence of glucose, under glucose-free conditions it reached a peak concentration at approximately 4 - 8 minutes and decreased beyond that time-point. The corresponding changes in contractile function shown in Figure 4.6 indicate that the onset of contracture development occurred at approximately 5 - 10 minutes after the introduction of glucose-free low-flow ischaemia. Therefore, contracture development occurred shortly after the decrease of glycolytic output.

4.2.5. Contracture development in the paced low-flow ischaemic heart perfused in the absence of glucose

The development of contracture in glucose-free low-flow ischaemic hearts may reflect varying degrees of ATP depletion in different areas of the myocardium rather than a uniform loss of energy reserves. Contracture development does not correlate with the loss of ATP in the ischaemic heart (van der Merwe et al., 1983). These findings were confirmed in the guinea-pig heart by measuring the ATP content of the low-flow ischaemic heart at the onset of contracture (section 4.2.6.1).

It was evident from the tissue concentrations of lactate (Table 4.1) that the reduction in the release of lactate into the perfusate during glucose-free low-flow ischaemia did not reflect retention of lactate within the tissue. However, contracture

developed in these hearts and this could restrict tissue perfusion, resulting in reduced lactate production. The consequences of contracture development (increased diastolic tension) on lactate release in the ischaemic heart were therefore investigated (section 4.2.6.2).

4.2.5.1. The metabolic characteristics of the glucose-free perfused low-flow ischaemic heart at the onset of contracture

The energy status of paced glucose-free low-flow ischaemic hearts was measured after a small degree of contracture had developed. The mean increase in diastolic tension was 7.4 ± 1.5% (n=5) and the mean sampling time was 7.3 ± 0.8 minutes after the start of the ischaemic period. This was similar to the time-course of contracture development observed in earlier experiments (see Figs 4.4b and 4.5b).

In comparison to paced normoxic hearts, both creatine phosphate and glycogen showed marked reductions to 4.6 ± 0.5 and 41.6 ± 5.6μmol/g dry wt but ATP concentrations were still relatively high at 11.1 ± 0.7μmol/g dry wt. The tissue lactate content was 17.3 ± 3.3μmol/g dry wt.

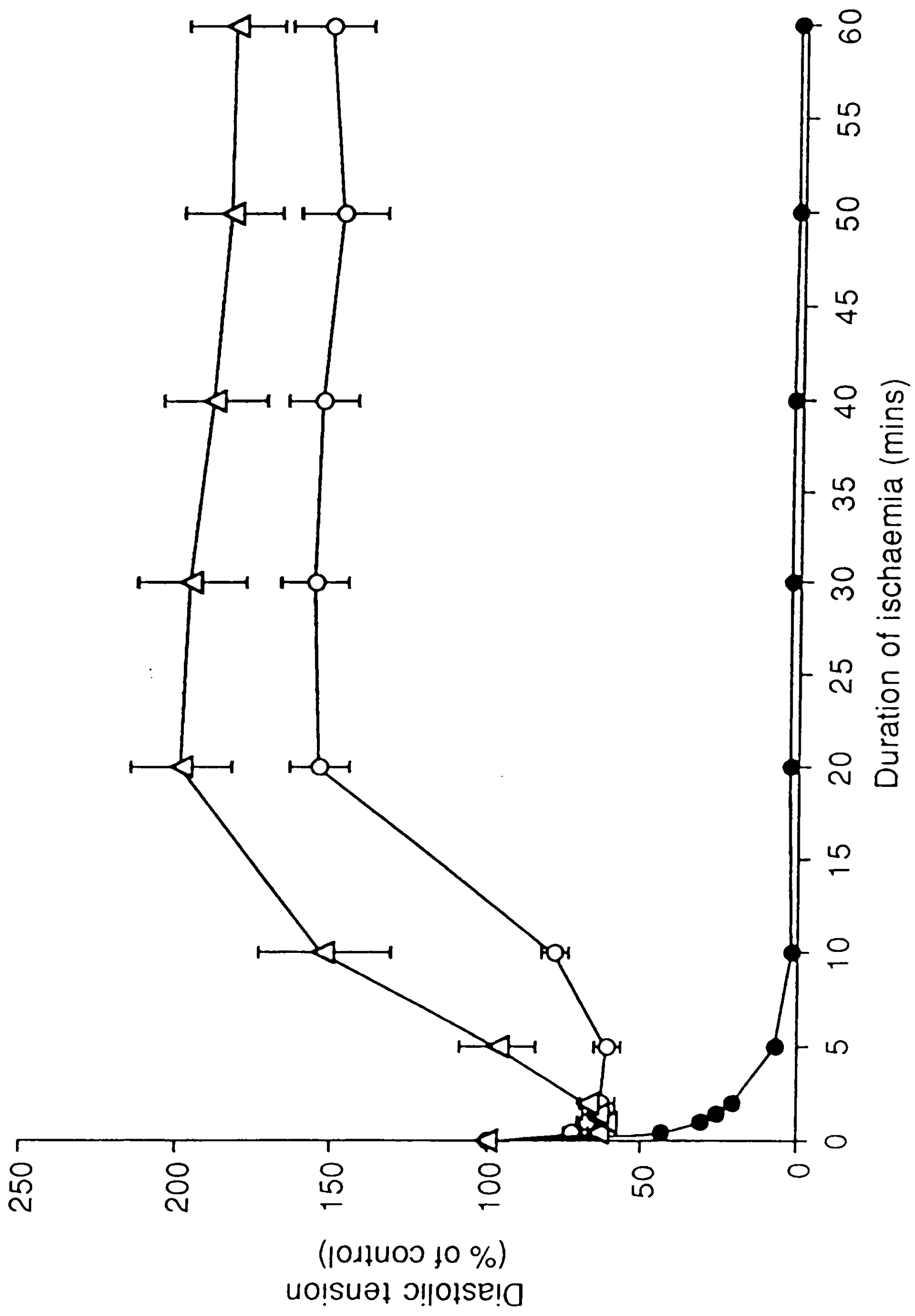
These results suggested that at the onset of contracture in these experiments, glycogen and creatine phosphate were available in amounts adequate to support ATP requirements. Contracture commenced in these experiments at a time when the total tissue ATP was high. However, the measurement of whole tissue ATP gives no indication as to the distribution of energy deprivation in the myocardium.

4.2.5.2. The potential consequences of contracture development in the paced perfused heart during low-flow ischaemia.

The relationship between contracture and glycolysis was further investigated by

Figure 4.8 The effect of glucose on contracture development during low-flow ischaemia in the paced perfused guinea-pig heart

This figure shows the effects of 60 minutes of low-flow ischaemia in the presence of glucose (n=4, closed circles), 60 minutes of glucose-free low-flow ischaemia (n=6, open circles) and 60 minutes of low-flow ischaemia which comprised 30 minutes of glucose-free low-flow followed by 30 minutes of low-flow in the presence of glucose (n=6, open triangles). Values represent changes in diastolic tension and are mean ± S.E. expressed as % of pre-ischaemic values.


Diastolic tension
(% of control)
0
50
100
150
200
250
Duration of ischaemia (mins)
0
5
10
15
20
25
30
35
40
45
50
55
60

studying lactate release during low-flow ischaemia. In the first set of experiments, the low-flow ischaemic period in glucose perfused hearts was extended to 60 minutes and the lactate released into the perfusate was collected in two separate samples between 0 - 30 minutes and between 30 - 60 minutes. The total lactate released during the 0 - 30 minute period was 402.6 ± 29.9μmol/g dry wt. During the 30 - 60 minute period, 443.5 ± 28.5μmol/g dry wt were released. This data showed that the glycolytic flux during both periods of ischaemia was similar and Figure 4.8 showed that no contracture development took place during the 60 minute ischaemic period.

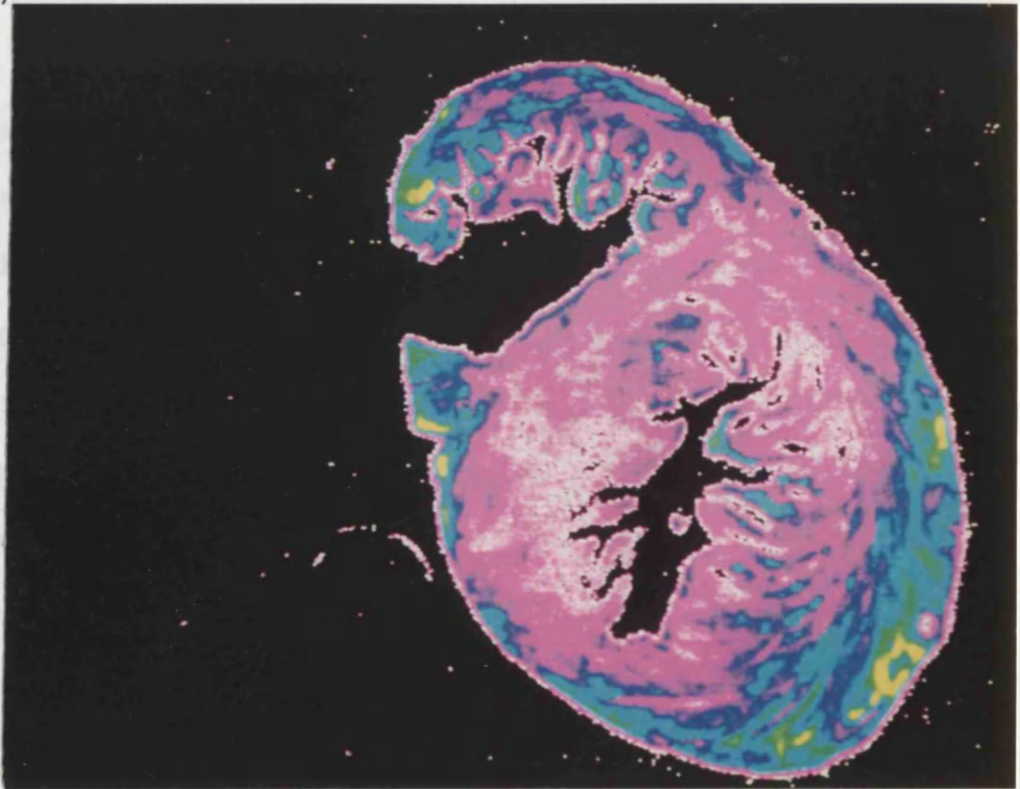
These results were compared with lactate release during two consecutive 30 minute periods of glucose-free low-flow ischaemia. The diastolic tension measurements made in this group are also shown in Figure 4.8. Total lactate release during the two 30 minute periods was 167.2 ± 9.6 and 20.0 ± 3.2μmol/g dry wt, respectively. This reduction of lactate production was associated with contracture development and was consistent with earlier findings that lactate production decreased during glucose-free low-flow ischaemia (see Figure 4.6).

Finally, the ability of the ischaemic heart to produce lactate after contracture had developed was investigated. Hearts were perfused for 30 minutes under glucose-free low-flow conditions, during which contracture developed, then while low-flow conditions were maintained glucose was re-introduced and perfusion continued for a further 30 minute period. The total lactate production during 30 minutes of glucose-free low-flow ischaemia was 106.5 ± 13.8μmol/g dry wt. During this period contracture developed to 195.9 ± 17.6% of control. Following the re-introduction of glucose to these hearts, lactate release during the remaining 30 minutes of low-flow ischaemia was 109.7 ± 15.8μmol/g dry wt. Although this represented an increase of approximately 5-fold compared to the lactate released during the corresponding period of glucose-free ischaemia, it was only 25% of the total amount released during the same period of low-flow ischaemia in the presence

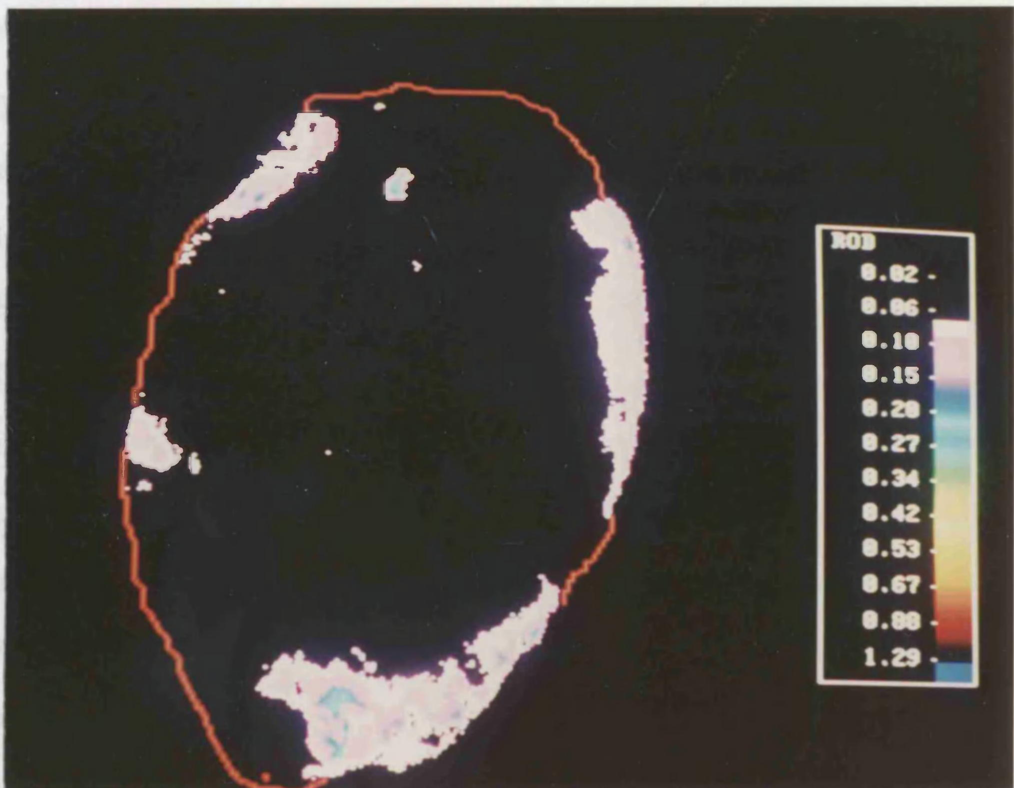
Figure 4.9 The effect of contracture development on myocardial perfusion during low-flow ischaemia with and without glucose in the paced perfused guinea-pig heart

The area of perfusion during low-flow ischaemia in the presence of glucose (Figure a) was compared with the area perfused after contracture development following glucose-free perfusion (Figure b); (see section 4.2.5.2). These photographs represent transverse sections of the myocardium after perfusion with 0.1% Evan's blue dye at the end of the ischaemic period. Each section was prepared histologically and the degree of perfusion was differentiated using an image analyser (Turnkey TK/20). In Figure a) the epicardial areas (blue/yellow) were perfused to a greater degree than the endocardium (pink). In Figure b) there is almost complete loss of perfusion.

a)



b)



of glucose. Despite the 5-fold increase in glycolysis, ischaemic contracture was not reduced (Figure 4.8).

The consequences of contracture on the degree of perfusion in the low-flow ischaemic heart are shown in Figure 4.9. This shows that the low-flow perfused area can be markedly reduced, with a corresponding increase in the no-flow area, after ischaemic contracture has developed. At least two factors can therefore account for the loss of lactate production after contracture: 1) under conditions of low perfusion pressure, contracture decreases perfusion and increases the no-flow ischaemic area leading to 2) restricted glycolytic capacity and subsequently increased cell death.

4.3 Lipid metabolism in the normoxic and ischaemic perfused heart

In the normoxic rat heart *in vitro*, endogenous triglyceride metabolism contributes to over 11% of the ATP production even in the presence of high concentrations of exogenous lipid; in the absence of exogenous lipid, triglycerides can become the major myocardial substrate (Saddik and Lopashuk, 1991). Glycerol derived from triglycerides (Shipp et al., 1964) is not re-metabolised by the heart and glycerol production has been used to demonstrate increased lipolysis (Myrmel *et al.*, 1991; van Bilsen *et al.*, 1989) in ischaemic hearts (Myrmel *et al.*, 1991). Accumulation of lipid intermediates, such as long-chain acylcarnitines (LCA), arising from impaired metabolism of both endogenous and exogenous lipids may result in increased ischaemic damage (Katz and Messineo, 1981; Knabb *et al.*, 1986). The prevention of LCA accumulation (e.g. by inhibition of carnitine palmitoyl transferase I (CPT-I)) may be beneficial in ischaemia (Paulson *et al.*, 1986). The availability of fatty acids can also decrease glucose utilitisation (Randle *et al.*, 1963; Weiss et al.,1989) and inhibition of glucose utilisation, as demonstrated in the previous sections, can markedly alter the myocardial response

to ischaemia Much of the published data has been derived from studies using rat tissue, therefore the following experiments have been performed to study some of the effects of fatty acid metabolism in the normoxic and ischaemic-perfused guinea-pig heart.

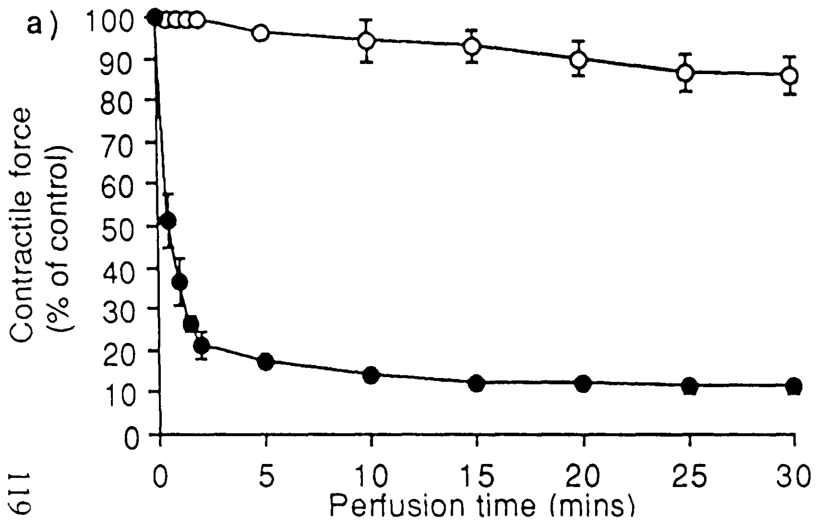
If endogenous lipids are metabolised in the isolated heart, then increased concentrations of LCA should be detected in the low-flow ischaemic heart perfused in the absence of exogenous fatty acids. This hypothesis was tested by comparing the LCA content of normoxic hearts and hearts subjected to 30 minutes of low-flow ischaemia with and without palmitate (4.3.1). The effect of lowering the glucose concentration from 11.7mM to 5mM on the response to palmitate was also investigated (4.3.2). Inhibition of CPT-I by etomoxir was studied in isolated mitochondria (4.3.3) and in the perfused heart (4.3.4). The potential effects of albumin - binding in perfusion studies are described in section 4.3.5. The effects of etomoxir on cardiac function and glycogen utilisation in normoxic hearts was examined (4.3.6).

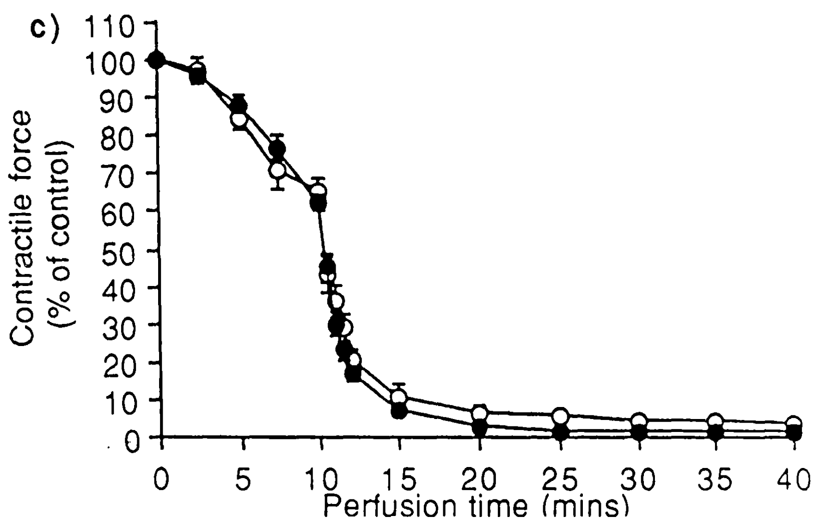
4.3.1. The LCA content of the paced normoxic and low-flow ischaemic perfused heart

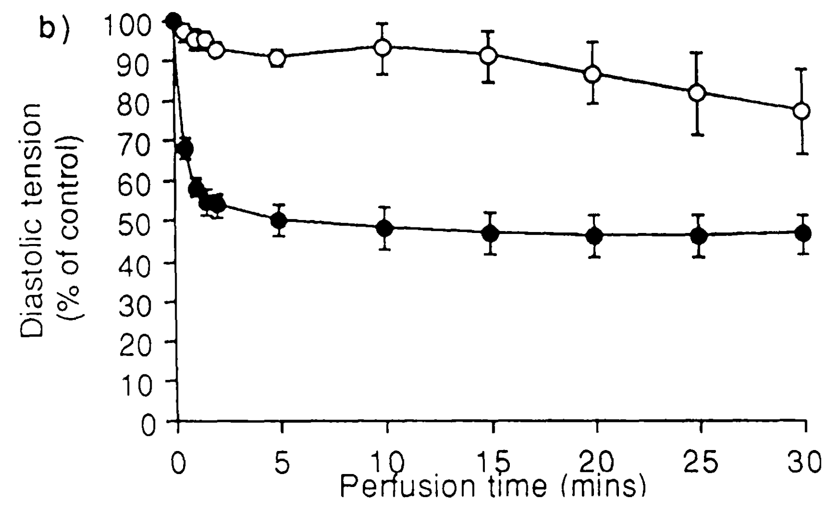
This study compared the LCA content of hearts perfused for 30 minutes under conditions of normoxia, low-flow ischaemia (no albumin/palmitate) and low-flow ischaemia with either 3% albumin alone (albumin group) or 1.5mM palmitate bound to 3% albumin (palmitate group). Several studies have used 1.5mM palmitate bound to 3% albumin to investigate the effects of fatty acids during ischaemia in the heart (Feuvray, 1981; Lopaschuk *et al.*, 1988) and the method of preparation of the palmitate: albumin solution is described in Section 2.3.2. Contractile activity and perfusate concentrations of creatine kinase were measured prior to and during the ischaemic period. The latter measurements were made on the basis that if fatty

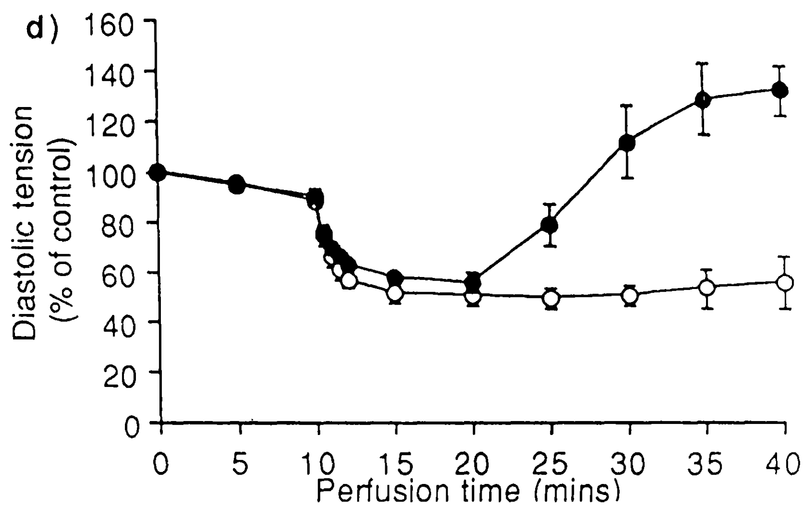
Figure 4.10 Comparison of the contractile changes in the paced normoxic and low-flow ischaemic guinea-pig heart in the presence of albumin or albumin-bound palmitate

The effects of normoxia (n=3, open circles) and low-flow ischaemia (n=3, closed circles) on contractile function are shown in Figures a) and b), respectively. The changes to contractile activity in hearts pre-treated for 10 mins with albumin (n=3, open circles) or palmitate (n=4, closed circles) prior to the 30 minute low-flow ischaemic period are shown in Figures c) and d), respectively. Albumin ± palmitate was also present in the perfusate during the ischaemic period. Values shown are mean ± S.E. expressed as % of the measurement made at the end of 30 minutes equilibration.


a)
Contractile force
(% of control)
Perfusion time (mins)


c)
Contractile force
(% of control)
Perfusion time (mins)


b)
Diastolic tension
(% of control)
Perfusion time (mins)


d)
Diastolic tension
(% of control)
Perfusion time (mins)

acids increased ischaemic damage in this model, this may be reflected in increased release of creatine kinase.

4.3.1.1 The effects of albumin and albumin-bound palmitate on contractile function in the paced perfused heart during low-flow ischaemia

Control measurements of contractile force and diastolic tension taken at the end of the equilibration period in the normoxic group were 1.0 ± 0.3 and 4.3 ± 0.1g, respectively and in the ischaemic group, the corresponding measurements were 0.9 ± 0.1 and 4.6 ± 0.3g. In the albumin group, the values were 1.1 ± 0.2 and 4.8 ± 0.4g and in the palmitate group were 0.8 ± 0.1 and 4.6 ± 0.3g, respectively. The pacing rate was 320 ± 29 beats/min in the normoxic group, 345 ± 4 beats/min in the ischaemic group, 341 ± 27 beats/min in the albumin group and 352 ± 15 beats/min in the palmitate group.

Contractile changes during normoxia and low-flow ischaemia are shown in Figure 4.10 (a) and Figure 4.10 (b).

Pre-treatment with albumin or palmitate prior to ischaemia caused a reduction in contractile force to 65.6 ± 3.2% and 62.3 ± 2.3% of control, respectively. The reason for this effect was unclear. There was no significant difference in the decrease in contractile force during ischaemia in the albumin and palmitate groups (Figure 4.10c).

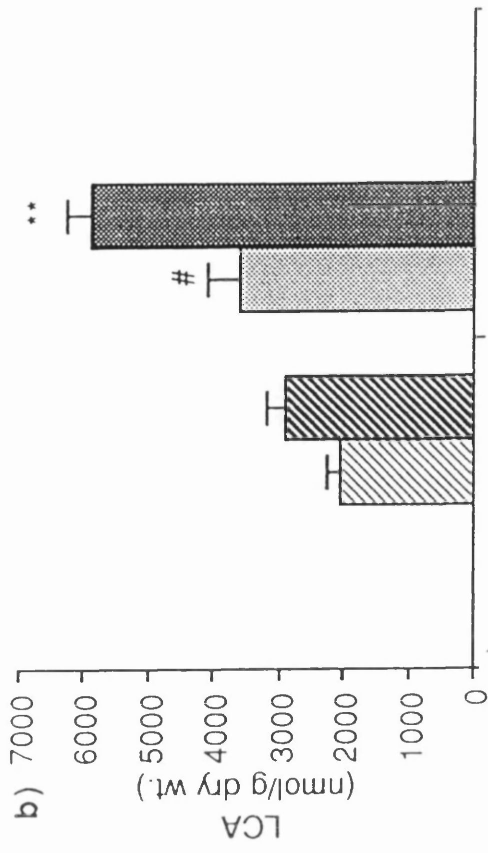
Diastolic tension was only slightly decreased during the pre-treatment period. While diastolic tension in the albumin group was 55.8 ± 10.7% of control after 30 minutes of ischaemia, it increased to 132.2 ± 9.7% of control in the palmitate group (Figure 4.10d).

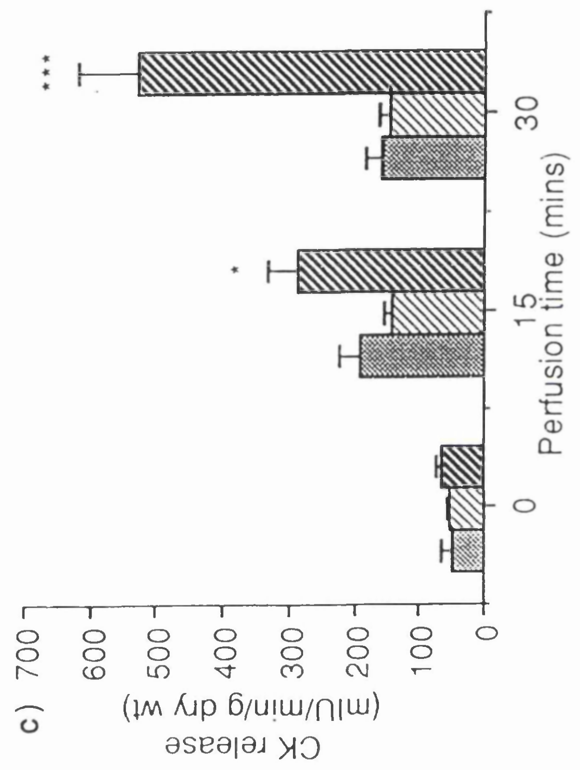
Figure 4.11 Long-chain acylcarnitine content and creatine kinase release in the paced normoxic, low-flow ischaemic and low-flow ischaemic guinea-pig heart perfused with albumin or albumin-bound palmitate

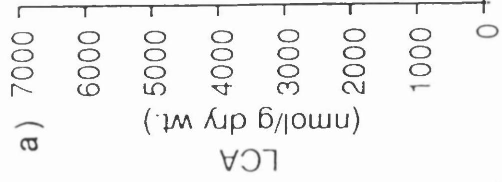
The tissue content of long chain acylcarnitine (LCA) after 30 minutes of normoxia (n=3, dark filled bars) or low-flow ischaemia (n=3, dark hatch bars) are shown in Figure a). Figure b) shows the LCA content of albumin-perfused normoxic (n=3, light hatch bars) and low-flow ischaemic hearts (n=7, dark hatch bars) compared to palmitate-perfused normoxic (n=3, light shaded bars) and low-flow ischaemic hearts (n=7, dark shaded bars).

Creatine kinase (CK) concentrations during low-flow ischaemia (shaded bars) and low-flow ischaemia with albumin ± palmitate (light and dark hatch bars, respectively) are shown in Figure c). The enzyme concentrations were measured in perfusate samples collected at the times indicated (14 -16 mins and 28 - 30 mins). Time 0 represents basal release prior to low-flow ischaemia. Values are mean ± S.E. expressed as mIU/min per g dry wt after conversion according to the flow rate/min divided by the dry tissue wt.

In figure b), statistical comparison of the albumin and palmitate groups was by unpaired t-test (# $p < 0.05$ compared to albumin control; **$p < 0.01$ compared to low-flow ischaemia + albumin). In figure c), the 15 and 30 mins values shown for low-flow ischaemia alone and for low-flow ischaemia in the presence of albumin were not significantly different. These values were significantly higher compared to time 0 ($p < 0.05$, not indicated). Enzyme release at 15 and 30 mins of low-flow ischaemia in the presence of palmitate was significantly higher compared to low-flow ischaemia ± albumin (*$p < 0.05$; ***$p < 0.001$)


b)
LCA
(nmol/g dry wt.)
7000
6000
5000
4000
3000
2000
1000
0
#
**


c)
CK release
(mIU/min/g dry wt)
700
600
500
400
300
200
100
0
0
15
30
Perfusion time (mins)
*
***


a)
LCA
(nmol/g dry wt.)
7000
6000
5000
4000
3000
2000
1000
0

4.3.1.2 The metabolic characteristics of the paced heart following perfusion with albumin and albumin-bound palmitate.

No significant difference was found in the LCA content of normoxic and low-flow ischaemic hearts (Figure 4.11a). This was also the case in normoxic and low-flow ischaemic hearts perfused with albumin (Figure 4.11b). The LCA content of the albumin-perfused hearts was higher than the hearts perfused without albumin but the reason for this was unclear since the albumin used in these studies was essentially fatty acid free (0.004%). Palmitate increased the LCA contents of both normoxic and ischaemic hearts (Figure 4.11b).

Measurement of creatine kinase release in the perfusate, prior to and at 15 and 30 minutes of ischaemia, indicated that an increase in the concentration of this enzyme had occurred during the ischaemic period. In the palmitate group, this increase was time-dependent and significantly greater than in the albumin group (Figure 4.11c).

The presence of LCA in the normoxic and ischaemic hearts perfused with and without albumin suggested that endogenous lipid was used as a substrate in the absence of exogenously supplied fatty acids. However, ischaemia alone did not result in LCA accumulation which was seen only in the presence of palmitate. The presence of palmitate during ischaemia was also associated with contracture development and increased release of creatine kinase.

4.3.2. The effects of albumin and albumin-bound palmitate during low-flow ischaemia in paced hearts perfused with 5mM glucose.

While the indices of ischaemic damage, described in section 4.9.4, indicated a greater degree of damage in the palmitate-perfused hearts, it could not be concluded that this was a direct consequence of elevated LCA. The availabilty of fatty acid is

known to affect glucose metabolism and this could account for the effects observed in these experiments. To determine whether the concentration of glucose could affect the response of the heart to low-flow ischaemia, a similar set of ischaemic experiments using 5mM instead of 11.7mM glucose in the presence of albumin or albumin-bound palmitate were performed.

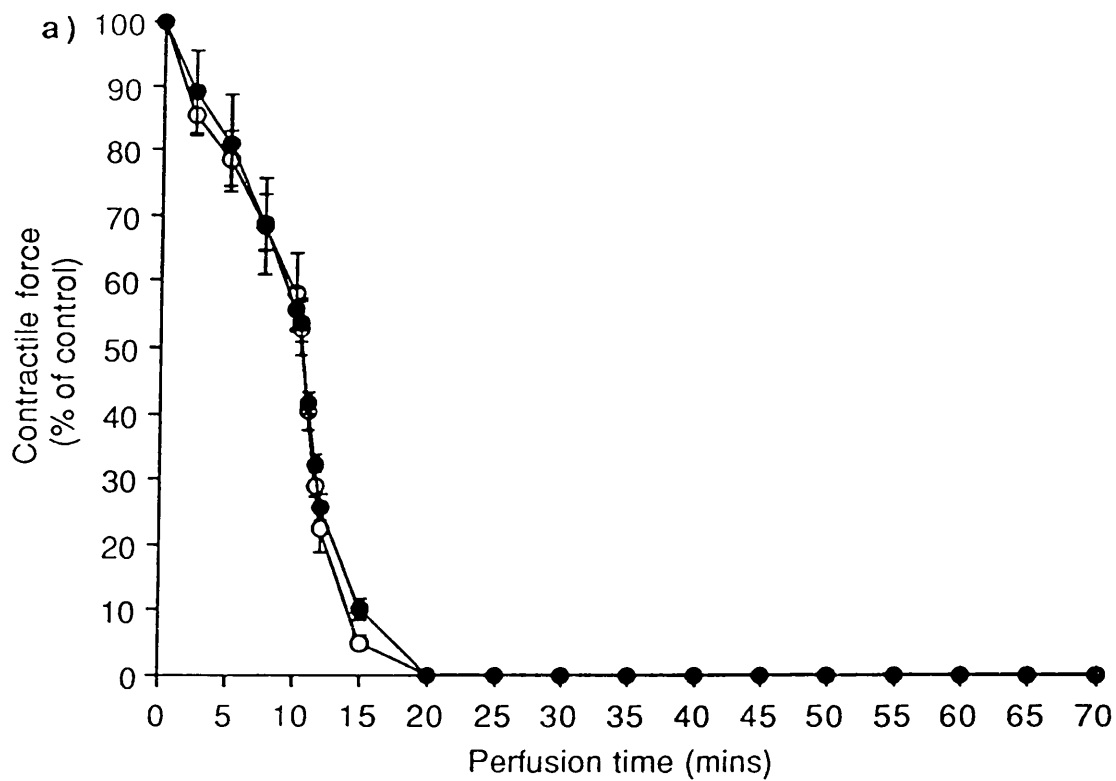
As in the previous experiments, the hearts were perfused with albumin (albumin group) or albumin-bound palmitate (palmitate group) for 10 minutes prior to ischaemia but in initial experiments some of the hearts fibrillated at this stage. To reduce the possibility of fibrillation, the hearts in these experiments remained unpaced during the equilibration period and the pre-treatment period. Electrical pacing was introduced after the onset of ischaemia at the time when the heart rate spontaneously slowed in response to ischaemia (see Figure 3.2). Thereafter, the hearts were paced at the rate recorded prior to the ischaemic period. This allowed the hearts to be paced at a slower rate than the usual 25% above the spontaneous rate. After the pre-treatment period, each heart was perfused for 60 minutes of low-flow ischaemia. The ischaemic period was extended from 30 to 60 minutes in an attempt to amplify the effects of ischaemia on contractility and enzyme release.

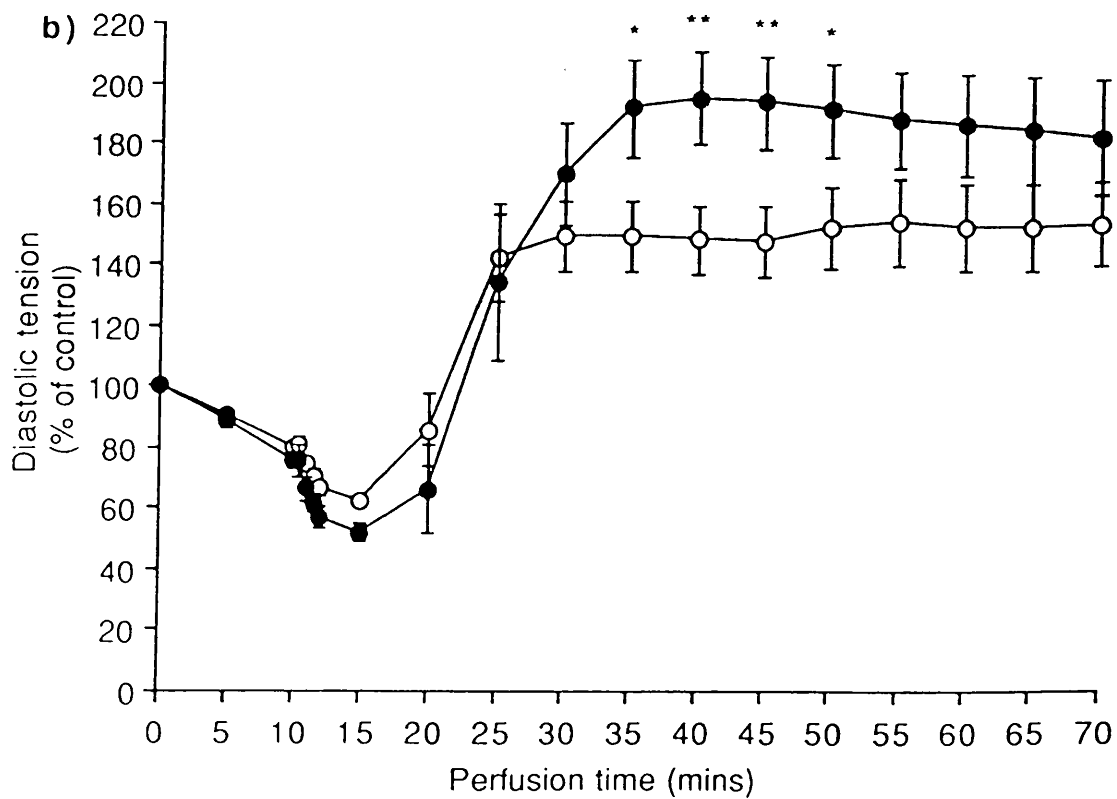
4.3.2.1 The effects of albumin and albumin-bound palmitate on contractile function during low-flow ischaemia in the paced heart perfused with 5mM glucose

The control values for contractile force measured at the end of the 30 minute equilibration period were 1.0 ± 0.1g (albumin group) and 1.2 ± 0.1g (palmitate group). Perfusion with albumin or palmitate during the 10 minutes pre-treatment period before low-flow ischaemia caused a decrease in contractile force to 58.3 ± 6.0% and 55.8 ± 3.1% of the control values, respectively (Figure 4.12a). Control measurements of diastolic tension were 4.5 ± 0.3g and 4.1 ± 0.3g, respectively. The diastolic tension was also slightly reduced during the albumin and palmitate pre-

Figure 4.12 Comparison of the contractile changes in the paced low-flow ischaemic guinea-pig heart perfused with albumin or albumin-bound palmitate in the presence of 5mM glucose

The perfusate concentration of glucose in these experiments was 5mM (see section 4.3.2). Each heart was also perfused with albumin or albumin-bound palmitate for 10 minutes prior to the low-flow ischaemic period. The effects of low-flow ischaemia in the presence of albumin (n=5, open circles) or albumin-bound palmitate (n=5, closed circles) on contractile function are shown in Figures a) and b), respectively. Albumin ± palmitate was also present in the perfusate throughout the ischaemic period. Values shown are mean ± S.E. expressed as % of the measurement made at the end of the 30 minutes equilibration period prior to perfusion with albumin ± palmitate. Statistical analysis comparing corresponding time-points in the albumin and palmitate groups was by analysis of variance and application of a 2-sided t-test (*p< 0.05; **p< 0.01).


a)
Contractile force
(% of control)
100
90
80
70
60
50
40
30
20
10
0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
Perfusion time (mins)


b)
Diastolic tension
(% of control)
220
200
180
160
140
120
100
80
60
40
20
0
* ** ** *
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
Perfusion time (mins)

treatment periods (Figure 4.12b). The pacing rate during ischaemia was 232 ± 10 beats/min in the albumin group and 243 ± 6 beats/min in the palmitate group.

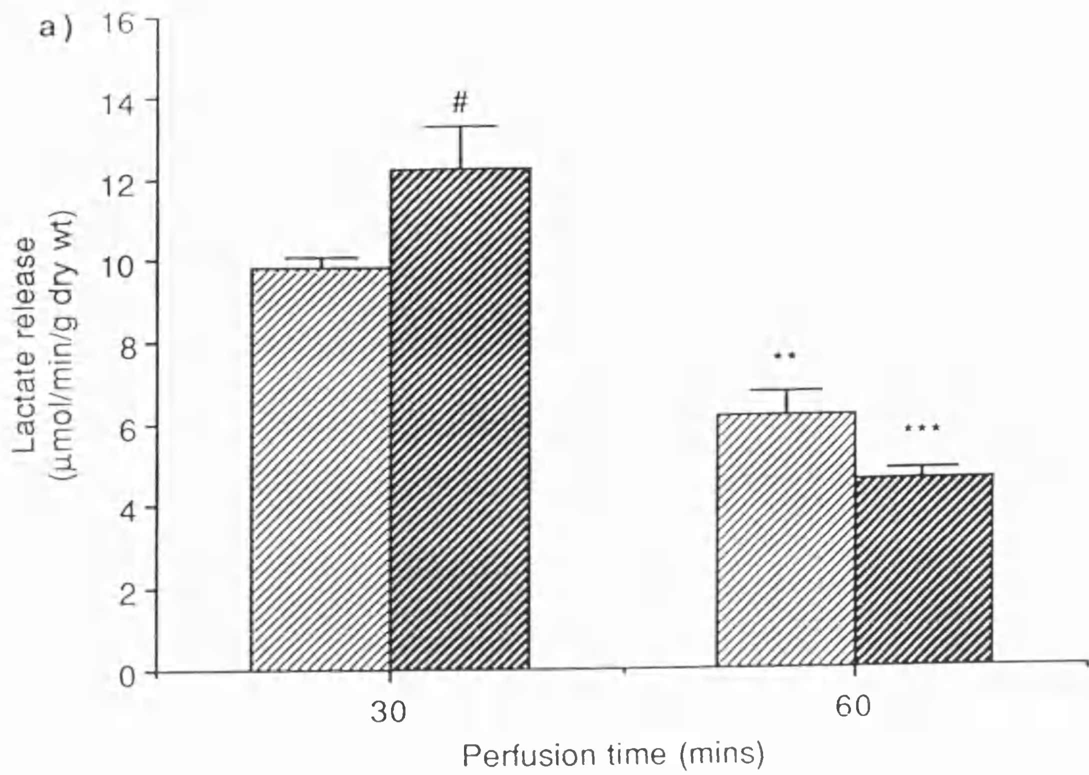
The loss of contractile force during low-flow ischaemia in the presence of 5mM glucose was greater than that observed with 11.7mM glucose. After 10 minutes, contractile force had ceased in both groups (Figure 4.12a). In addition, contracture developed in both groups during the ischaemic period. This was in contrast to the effects of low-flow ischaemia in the presence of 11.7mM glucose where contracture development was observed in the palmitate group alone (section 4.9.4). In the present study, contracture development was greater in the palmitate group and reached statistical significance between 25 - 40 minutes of ischaemia. This effect was most pronounced after 30 minutes of ischaemia when diastolic tension in the palmitate group was 194.6 ± 15.6% of control ($p < 0.01$) compared to 147.9 ± 11.0% of control in the albumin group (Figure 4.12b).

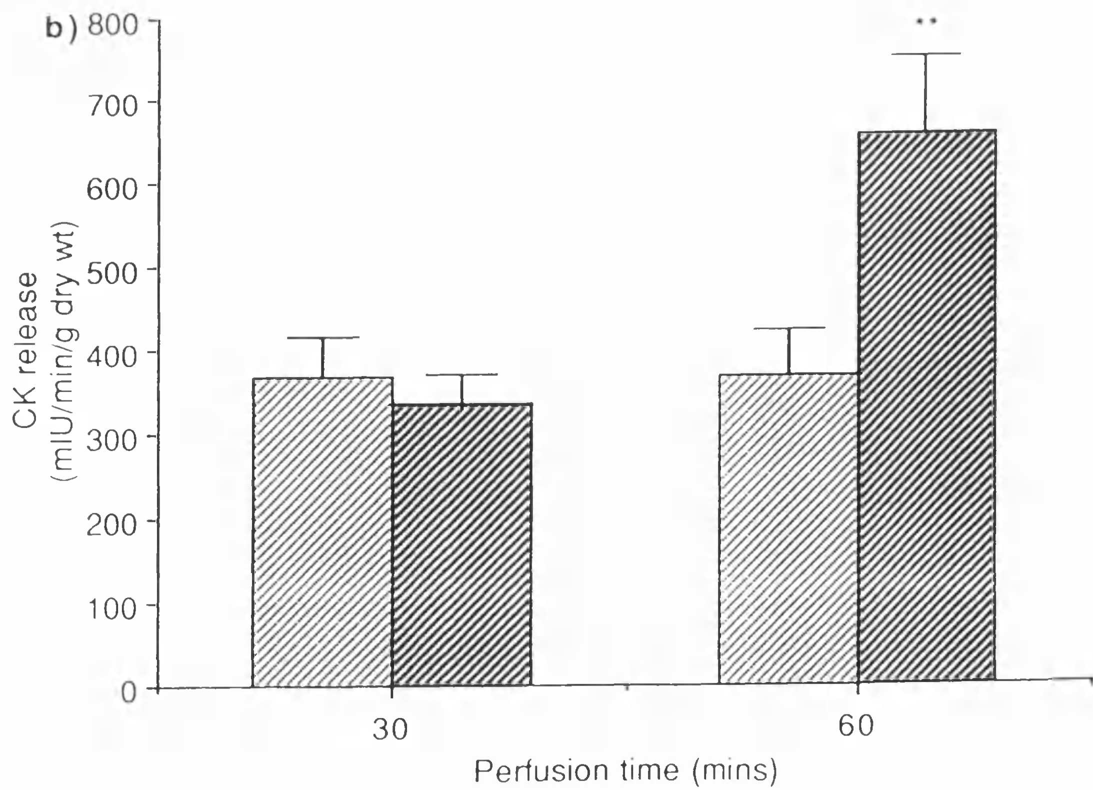
4.3.2.2 The effects of albumin and albumin-bound palmitate on the metabolic characteristics of the paced low-flow ischaemic heart perfused with 5mM glucose

Control LCA concentrations, measured at the end of the 10 minute pre-treatment period, were 1863 ± 179nmol per g dry wt (albumin) and 3094 ± 292nmol per g dry wt (palmitate). These values were similar to the previous study (see Figure 4.11). At the end of 60 minutes of ischaemia, the LCA concentrations in the albumin group were 3415 ± 404nmol per g dry wt and 5539 ± 242nmol per g dry wt in the palmitate group. This showed that although LCA concentrations in the palmitate group were higher after low-flow ischaemia, this increase was partly due to the increased LCA concentration observed during the pre-treatment period. The myocardial LCA content after 30 minutes (see Figure 4.11) of ischaemia was not significantly different from 60 minutes of ischaemia. This suggested that further accumulation of LCA had not taken place during the 30 - 60 minute period although

Figure 4.13 Lactate and creatine kinase release in the paced low-flow ischaemic guinea-pig heart perfused with albumin or albumin-bound palmitate in the presence of 5mM glucose

Lactate release from low-flow ischaemic hearts (see Figure 4.12) perfused with albumin (n=5, light hatch bars) or albumin-bound palmitate (n=5, dark hatch bars) is shown in Figure a). Creatine kinase (CK) release is shown in Figure b). All measurements were made from the total perfusate collected between 0 - 30 minutes and 30 - 60 minutes of ischaemia. Values are mean ± S.E. and are expressed as described in Figure 4.16 legend. Statistical analysis comparing albumin and palmitate values was by analysis of variance and application of a 2-sided t-test. (Figure a) # $p < 0.05$, ** $p < 0.01$ compared to 30 mins albumin, *** $p < 0.001$ compared to 30 mins palmitate; Figure b) ** $p < 0.01$ compared to both 30 mins values and to the 60 mins albumin value).


a)
16
14
12
10
8
6
4
2
0
Lactate release
(µmol/min/g dry wt)
#
**
***
30
60
Perfusion time (mins)


b)
800
700
600
500
400
300
200
100
0
CK release
(mIU/min/g dry wt)
**
30
60
Perfusion time (mins)

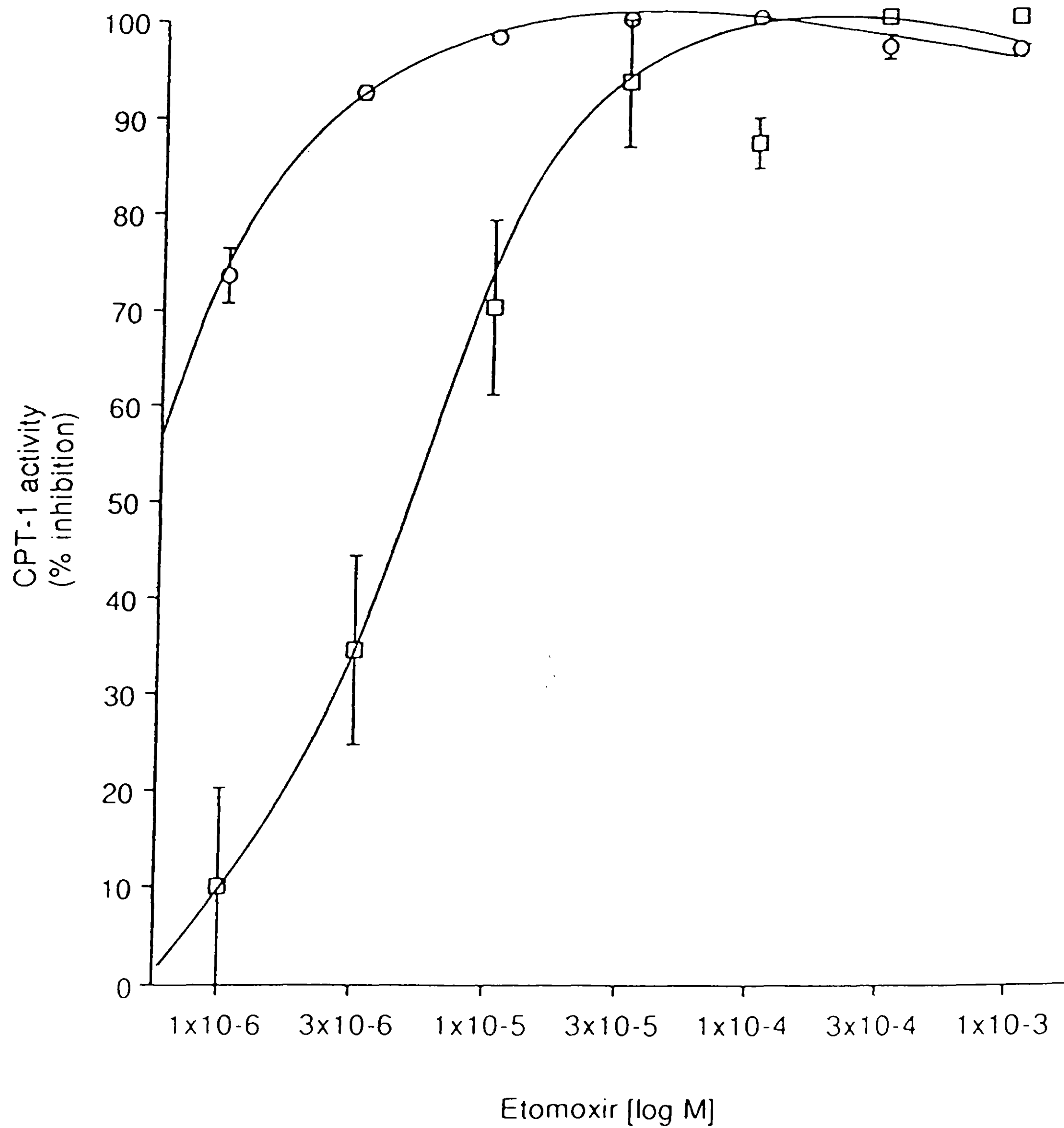
the possible loss of LCA into the perfusate could not be excluded in these experiments.

During the 0 - 30 minute ischaemic period, lactate production in the presence of palmitate was, in fact, significantly greater ($p < 0.05$) than in the albumin perfused group (Figure 4.13a). Therefore, there was no evidence from these results that the presence of palmitate had reduced glycolysis during 30 minutes of ischaemia. Lactate release was not sustained in either group and subsequently decreased during the 30 - 60 minute ischaemic period. Although these latter values represented mean reductions of 38% (albumin) and 63% (palmitate) from the 0 - 30 minutes values, this difference was due to the higher lactate concentration released from the palmitate-treated hearts during the 0 - 30 minute ischaemic period. Therefore, the effects of palmitate on lactate release were unclear in these experiments. The results did suggest however, that 5mM glucose, unlike 11.7mM (see section 4.2.5.2), did not adequately support ischaemic metabolism since lactate release decreased over the 60 minute period .

Although creatine kinase release in the albumin and palmitate groups was similar during 0 - 30 minutes of ischaemia, it was significantly greater in the palmitate group during the 30 - 60 minute ischaemic period (Figure 4.13b). This showed that ischaemic damage was increased when palmitate was present. Although the effect of palmitate during ischaemia in this model was associated with elevated LCA concentrations, a causal link between the two events could not be definitely established. Subsequently, it was considered that if etomoxir, an inhibitor of fatty acid oxidation (Wolf et al., 1981), could be shown to reduce LCA concentrations and ischaemic damage in palmitate-perfused low-flow ischaemic hearts, this would give greater support for the hypothesis that elevated LCA and ischaemic damage are linked.

Figure 4.14 The effect of etomoxir on palmitoylcarnitine transferase 1 (CPT-1) activity in rat and guinea-pig heart mitochondria

The relative sensitivities of CPT-1 activity to etomoxir in rat (n=3, open circles) and guinea-pig (n=3, open squares) heart mitochondria were measured as described in section 4.3.3. Values shown are mean ± S.E. and are expressed as a % of the activity measured in untreated control mitochondria.



CPT-1 activity
(% inhibition)
100
90
80
70
60
50
40
30
20
10
0
1x10-6
3x10-6
1x10-5
3x10-5
1x10-4
3x10-4
1x10-3
Etomoxir [log M]

4.3.3. Inhibition of myocardial CPT-I by etomoxir in the isolated mitochondria preparation

Before studies into the effects of etomoxir in the heart were performed, the activity of this compound as an inhibitor of carnitine palmitoyltransferase I (CPT-1; Selby and Sherratt, 1989), was investigated. This was done using an assay system which had already been established in this laboratory. However, this assay system involved the use of mitochondria prepared from rat heart and the first experiment was to determine the activity of etomoxir using guinea-pig heart mitochondria. A full description of the assay procedure is given in Section 2.3.4 and the assistance of Dr. Katrina Wyatt (Syntex) with these studies is gratefully acknowledged.

The ability of etomoxir to inhibit CPT-I in rat and guinea-pig isolated mitochondria was studied over the concentration range 10^{-8} to 10^{-3} M. The assay system was based on the amount of palmitoyl-[^{3}H]-carnitine produced in 5 minutes and the degree of inhibition, at each concentration of etomoxir investigated, was determined by a comparison with control mitochondria. From this data a pIC_{50} value for etomoxir in both preparations was calculated.

A comparison of the relative sensitivities of rat and guinea-pig myocardial CPT-I to etomoxir is shown in Figure 4.14 and it was found that the inhibitory potency of etomoxir was greater in rat (pIC_{50} 6.32) than in guinea-pig mitochondria (pIC_{50} 5.12). As a result, 10μM etomoxir was used for further studies in the guinea-pig heart.

4.3.4. Evidence for CPT-I inhibition by etomoxir in the paced normoxic-perfused heart

The method of measuring CPT-I activity using isolated mitochondria was modified to demonstrate that when etomoxir, which is an irreversible inhibitor of

CPT-I (Selby and Sherratt, 1989), was perfused into the heart, the CPT-I enzyme was inhibited. Each heart was perfused with 10μM etomoxir for 30 minutes and the mitochondria were isolated. The CPT-I activity was measured as before and the changes in the activity of the enzyme in each group investigated are shown below.

Six groups of hearts (n=3) were used:

	% CPT-I activity
1) Normoxia	100
2) Normoxia + etomoxir	44
3) Normoxia + 3% albumin	100
4) Normoxia + 3% albumin + etomoxir	88
5) Normoxia + albumin : palmitate (1.5mM)	100
6) Normoxia + albumin : palmitate (1.5mM) + etomoxir	87

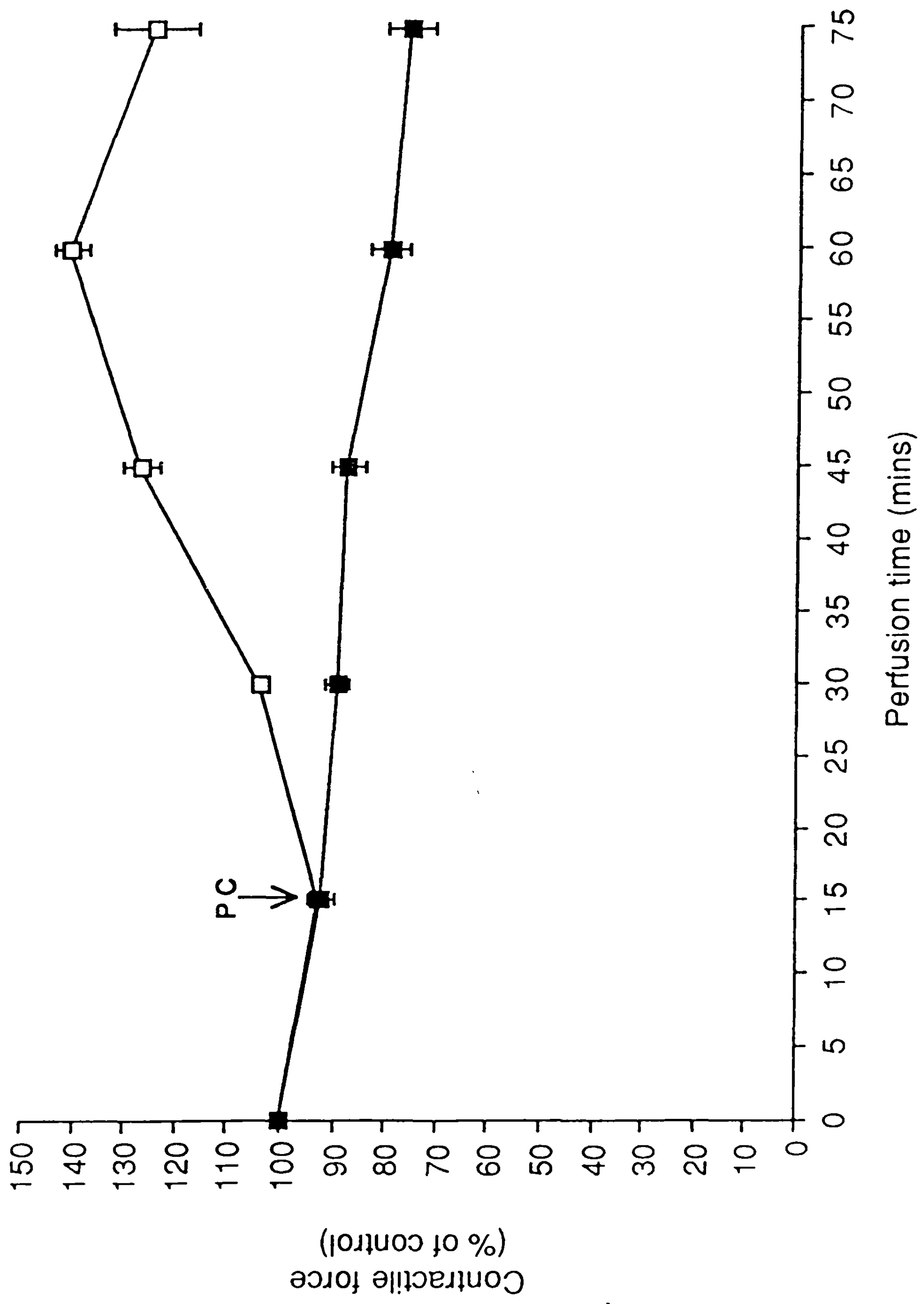
These results showed that the potency of etomoxir as an inhibitor of the CPT-I enzyme in the intact heart was affected by the presence of albumin in the perfusion solution. This observation suggested that studies involving the effects of drugs in albumin : palmitate-perfused preparations may underestimate the potency of those drugs. Conclusions drawn from such studies should therefore be viewed with caution.

4.3.5. The potential effects of albumin-binding in the perfused heart studies.

The degree to which LCA accumulation can increase ischaemic damage is uncertain because intracellular fatty acid binding proteins exist (Fournier *et al.*, 1983) and these may lower the free intracellular concentration of fatty acid

Figure 4.15 The effect of albumin on the positive inotropic activity of palmitoylcarnitine in the paced superfused guinea-pig papillary muscle preparation

The contractile response to 10μM palmitoylcarnitine (PC) in the absence of albumin (n=5, open squares) was compared with the response to PC in the presence of 0.02% albumin (n=6, closed squares). Tissues were pre-treated with either albumin or PSS for 15 minutes prior to the addition of PC. Values shown are mean ± S.E. expressed as % of the contractile measurement made prior to the pre-treatment period.



Contractile force
(% of control)
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
P C
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
Perfusion time (mins)

intermediates. Although it was not possible to demonstrate this interaction by intracellular methods, the effect of albumin-binding on the response of the guinea-pig papillary muscle to exogenous palmitoyl carnitine was investigated.

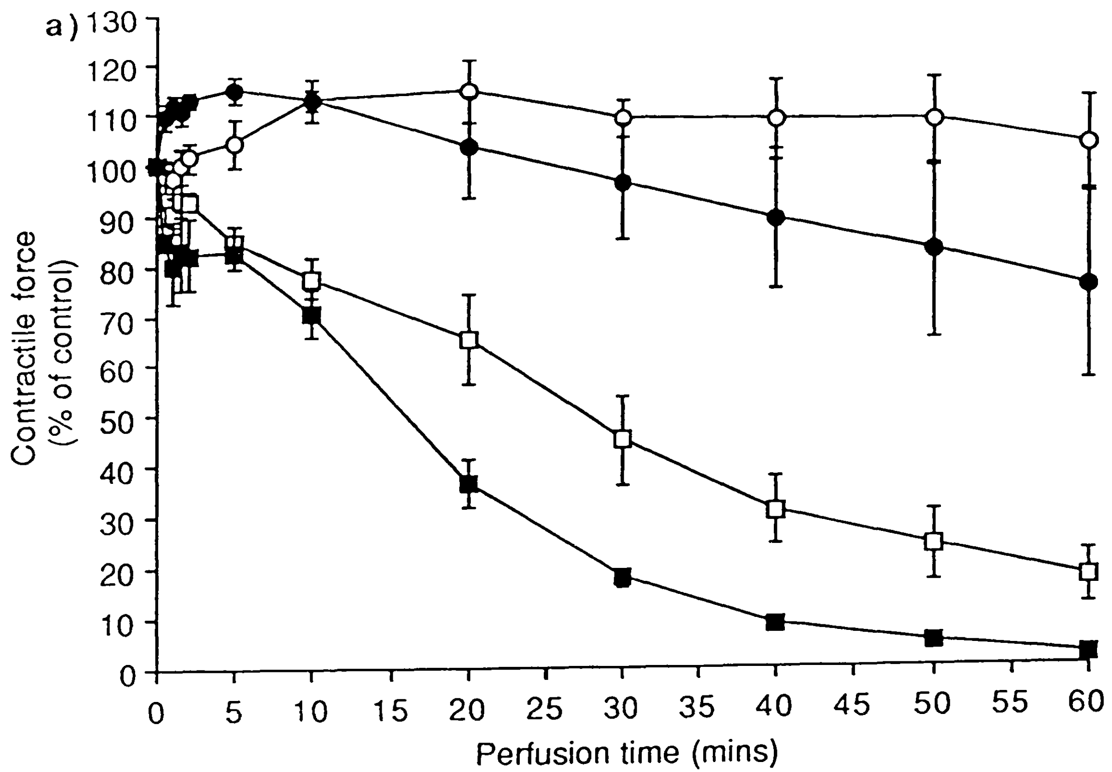
In previous studies, palmitoyl carnitine has been shown to increase contractile force in the guinea-pig papillary muscle preparation (Clarke *et al.*, 1990). The papillary muscles were set up as described in Section 2.3.2 and at the end of the equilibration period were superfused with either PSS (control group) or 0.02% albumin for 15 minutes. After this time, both the control group and the albumin group were perfused with 10μM palmitoyl carnitine for 60 minutes. The albumin was present throughout the 60 minute period. Since 3% albumin was used to bind 1.5mM palmitate in the previous experiments, the concentration of albumin used to bind 10μM palmitoyl carnitine experiments was scaled down to 0.02%. Changes to contractile force were measured every 15 minutes and the results were expressed as % of the contractile force measured at the end of the equilibration period.

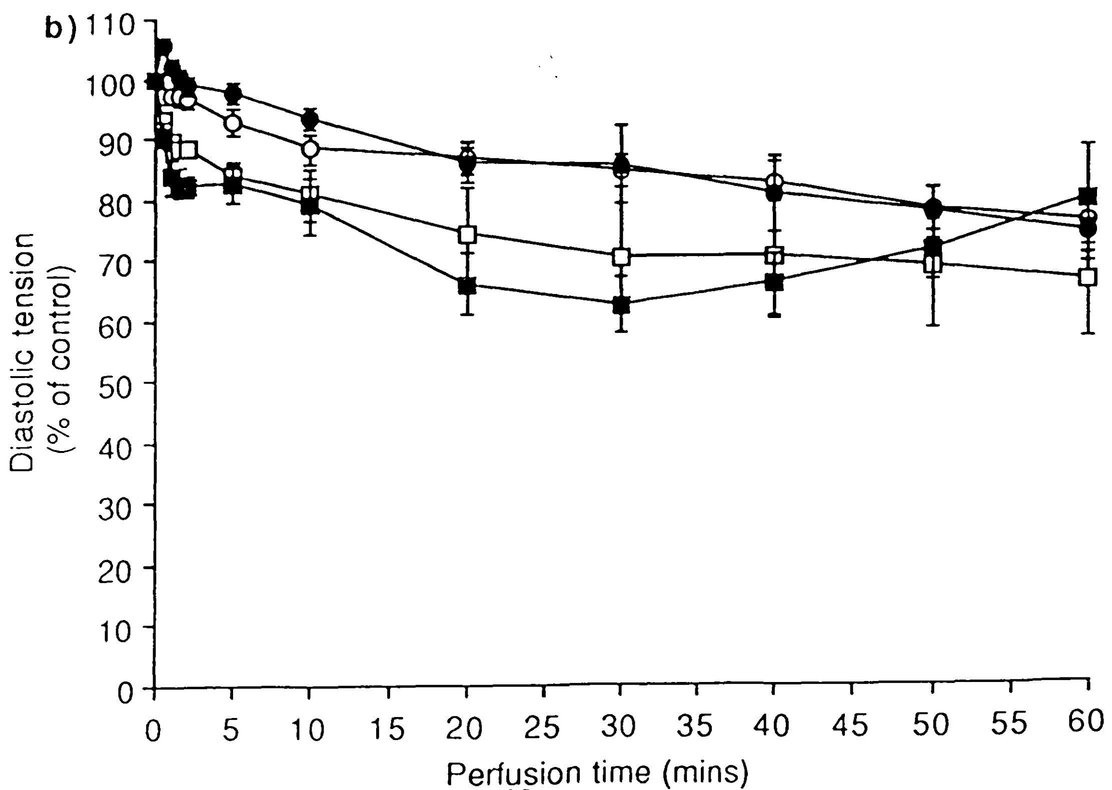
4.3.5.1. The effects of albumin on the positive inotropic response to palmitoyl carnitine in the guinea-pig papillary muscle preparation.

Contractile force increased to 141.2 ± 3.2% of control in the presence of 10μM palmitoyl carnitine. In contrast, palmitoyl carnitine in the presence of albumin, had no positive inotropic effect. This suggested that the interaction between palmitoyl carnitine and albumin had prevented the effect on contractile force (Figure 4.15). This supported the theory that binding proteins could alter the potential effects of LCA in the heart. In the time available it was not possible to resolve the problem of albumin : etomoxir binding and this prevented further studies of the effects of etomoxir in the palmitate-perfused ischaemic heart. However, the effect of etomoxir on carbohydrate utilisation was investigated further in the normoxic heart.

Figure 4.16 The effects of etomoxir on contractile activity in the paced guinea-pig heart during normoxic perfusion with and without glucose

This figure shows the changes to a) contractile force and b) diastolic tension in glucose (11.7mM, n=4, open circles), glucose-free (n=4, open squares), glucose + 10μM etomoxir (n=4, filled circles) and glucose-free + 10μM etomoxir (n=4, filled squares) perfused hearts. Values shown are mean ± S.E. expressed as a % of the measurements made at the end of the equilibration period and prior to each treatment.


a)
Contractile force
(% of control)
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
45
50
55
60
Perfusion time (mins)


b)
Diastolic tension
(% of control)
110
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
45
50
55
60
Perfusion time (mins)

4.3.6. The effects of etomoxir in the normoxic-perfused heart

In previous experiments (see section 4.3.1.2), the presence of LCA in isolated hearts perfused in the absence of exogenous fatty acids suggested that endogenous lipids may be used as substrates. The aim of these experiments therefore, was to investigate whether CPT-I inhibition could cause an increase in glycogen utilisation in the normoxic heart.

These experiments were performed using normoxic hearts perfused with and without 11.7mM glucose. In the glucose-free experiments, glucose was replaced by equimolar mannitol in order to restrict the substrate utilisation to endogenous lipid and glycogen. The hearts were unpaced to avoid possible fibrillation but the glucose-free perfusion time was extended to 60 minutes in an attempt to increase the degree of endogenous substrate utilisation.

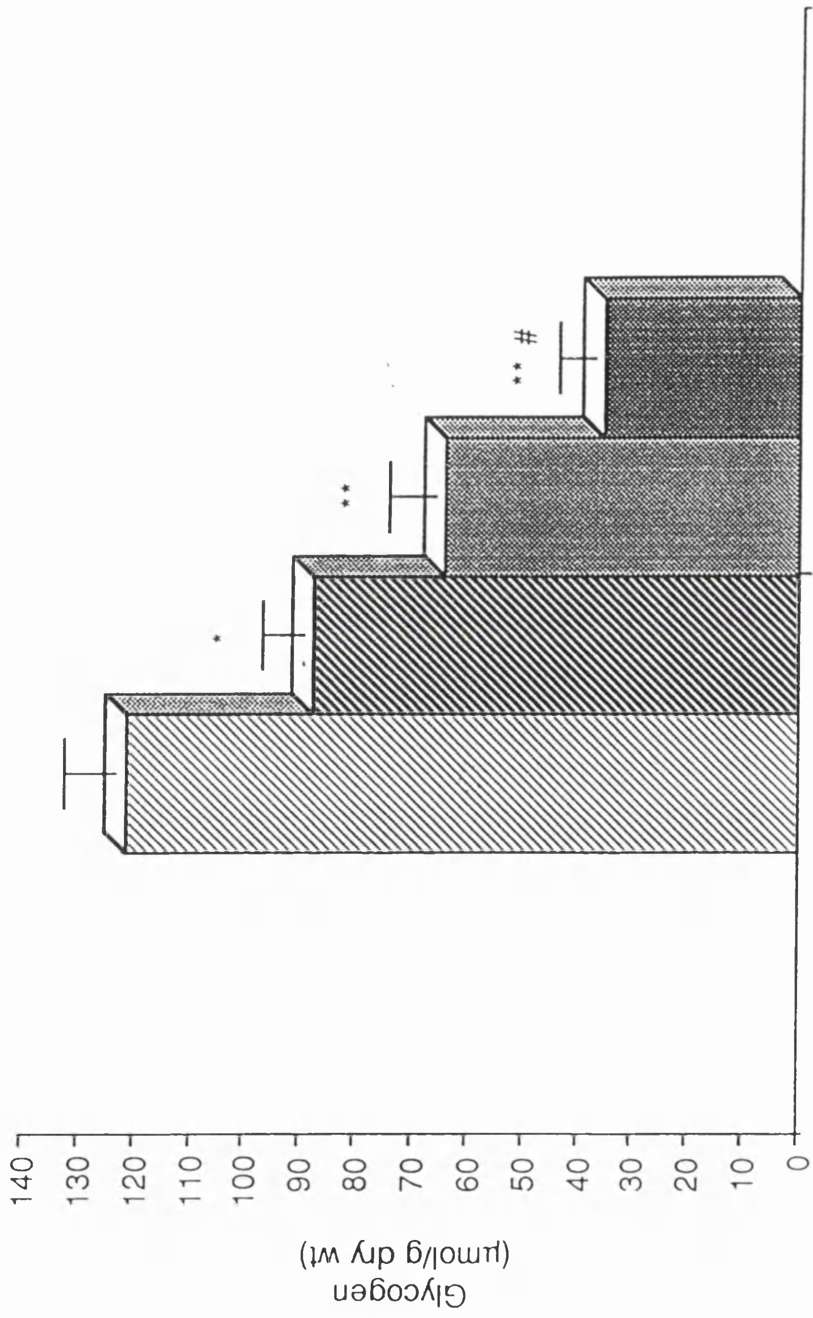
4.3.6.1. The effects of etomoxir on contractile function in the unpaced normoxic heart perfused with and without glucose

Contractile force in normoxic glucose-perfused hearts was 1.8 ± 0.5g at the start of the 60 minute perfusion period. The contractile force was relatively stable throughout the perfusion period. Prior to perfusion with etomoxir, the contractile force in a second group of hearts was 1.5 ± 0.3g. Etomoxir in the presence of glucose caused a small but non-significant decrease of contractile force to 74.7 ± 18.5% of control over 60 minutes (Figure 4.16a).

Contractile force prior to glucose-free perfusion was 1.5 ± 0.1g. Subsequently, glucose-free perfusion for 60 minutes caused a decrease in contractile force to 17.3 ± 5.4% of control. Prior to glucose-free perfusion in the presence of 10μM etomoxir, the contractile force in a second group of hearts was 1.1 ± 0.1g. When 10μM etomoxir was perfused under glucose-free conditions, a

Figure 4.17 The effects of etomoxir on glycogen levels in the paced guinea-pig heart during normoxic perfusion with and without glucose

The figure shows the changes to glycogen levels in glucose (11.7mM, n=4, light hatch bars), glucose + 10μM etomoxir (n=4, dark hatch bars), glucose-free (n=4, light shaded bars) and glucose-free + 10μM etomoxir (n=4, dark shaded bars) perfused hearts (see Figure 4.16). Values shown are mean ± S.E. and statistical comparison to glucose-perfused control values was by analysis of variance and application of Dunnett's t-test (*$p < 0.05$; **$p < 0.01$). Comparison of the glucose-free control group and the glucose-free + etomoxir group was by unpaired t-test (# $p < 0.05$).


Glycogen
(µmol/g dry wt)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
*
**
** #

greater decrease of contractile force, which was significant at all time-points beyond 10 minutes, was observed and after 60 minutes, contractile force had been reduced to 2.2 ± 0.8% of control (Figure 4.16a).

Changes to diastolic tension were not significantly different in these groups (Figure 4.16b) and there were no significant changes in the spontaneous heart rates during the perfusion periods.

4.3.6.2. The effects of etomoxir on the glycogen content of the unpaced normoxic heart perfused with and without glucose.

After 60 minutes, the control glycogen content of the glucose-perfused hearts was 121.4 ± 9.2μmol per g dry wt (Figure 4.17). In the presence of etomoxir, this concentration was significantly reduced to approximately 73% of control. In the glucose-free perfused group, the glycogen concentration was approximately 50% of control which was compatible with increased glycogen utilisation in the absence of exogenous glucose. The glycogen concentration in glucose-free hearts perfused with etomoxir was further reduced to approximately 30% of control. These results showed that etomoxir increased glycogen utilisation in both glucose and glucose-free perfused hearts.

The increased use of glycogen under glucose-free conditions could be explained by etomoxir-induced inhibition of lipid metabolism and this would support the theory that lipid may contribute to energy production in the perfused heart under normoxic conditions. However, in the presence of glucose, it might be expected that this loss of lipid substrate would be compensated for by increased glucose utilisation and Lopashuk *et al.* (1988) have proposed that etomoxir increases glucose utilisation in the heart. However, since glycogen utilisation in the presence of etomoxir was similar in both glucose and glucose-free perfused hearts in these experiments, there was no evidence that the presence of glucose altered the effects

of etomoxir.

4.4 DISCUSSION

In this chapter, the influence of the two major substrates, glucose and fatty acids, on the myocardial response to hypoxia and ischaemia have been studied. It is well documented that the concentration of fatty acids available to the heart can lead to a reduction of glucose metabolism and in this thesis, the potential effects of reducing glucose metabolism have been demonstrated in normoxic, hypoxic and ischaemic hearts.

It is clear from these studies that any reduction of glucose availability will result in increased ischaemic damage. In these experiments, a consistent response associated with the lowering of the glucose concentration was contracture development. This response was observed under glucose-free hypoxic conditions, a model in which glycolysis is not inhibited by accumulating metabolites and lactate. Therefore, even under conditions allowing glycolysis, maintenance of normal diastolic tension is limited by the availability of glycogen. The initial studies in this chapter examined the potential of glycogen to support normoxic function by removing the exogenous supply of glucose.

In the paced normoxic heart, the loss of contractile function over 90 minutes of glucose-free perfusion was associated with marked depletion of glycogen. In contrast, lactate efflux data suggested that the use of glycogen during low-flow ischaemia was relatively rapid under glucose-free conditions. In these experiments, glycogen was markedly reduced and the initial high rate of lactate efflux was not sustained in comparison to glucose-perfused low-flow ischaemic hearts. Contracture developed under glucose-free conditions in hypoxia and low-flow ischaemia and also in no-flow ischaemia. All of these conditions were associated with reduced glycolysis.

Although it is unclear whether the initiation of contracture development is loss of ATP or an increase in intracellular [Ca^{++}] (Lipasti et al., 1984), both of these events would be important in contracture development during ischaemia. Acidosis and the subsequent increase of intracellular [H^{+}] can displace Ca^{++} from the troponin binding site (Katz, 1973). When Ca^{++} binds to troponin, the inhibitory action of troponin on actin is removed leading to contraction. Therefore, if Ca^{++} binding is inhibited by [H^{+}], it could be argued that the result would be a permanent actin-myosin complex which could account for contracture development especially if the ATP concentration is low. However, in these studies no correlation between contracture and acidosis was found since contracture developed in paced hearts with high tissue lactate (no-flow ischaemia) and low tissue lactate (glucose-free hypoxia and glucose-free low-flow ischaemia). On the other hand, a high degree of ATP depletion was common to all of these hearts. The fact that contracture has been detected when the ATP concentration of whole tissue samples is relatively high can be explained by a variation in the distribution of ischaemia under low-flow conditions. This has been shown clearly in Figure 4.9a which indicates a higher degree of endocardial ischaemia.

Figure 4.9b shows the consequences of contracture development on the low-flow perfused heart. Contracture under these conditions exacerbates ischaemia by increasing the no-flow ischaemic area.

It was of interest therefore that contracture development was observed in the presence of glucose only when the glucose concentration was reduced from 11.7mM to 5mM or when palmitate was present. The observations with palmitate supported the theory that the presence of fatty acids could increase the effects of ischaemia. Whether this was due to a reduction of glucose uptake or the accumulation of fatty acid metabolites would require more investigation. Either explanation could account for the effects of palmitate in the low-flow ischaemic studies. However, contracture development during no-flow ischaemia cannot result

from the action of fatty acid intermediates since the oxidation of fatty acids would be completely inhibited. This is supported by the finding that fatty acid intermediates do not accumulate under no-flow conditions (Paulson *et al.*, 1984). During low-flow ischaemia, residual flow may allow some degree of oxidative metabolism to continue. Under these conditions, fatty acid metabolism would inhibit glucose oxidation and increased oxygen utilisation (Lopaschuk et al., 1990). These factors coupled with the accumulation of lipid intermediates (Katz and Messineo, 1981) could increase ischaemic cell damage.

CHAPTER FIVE

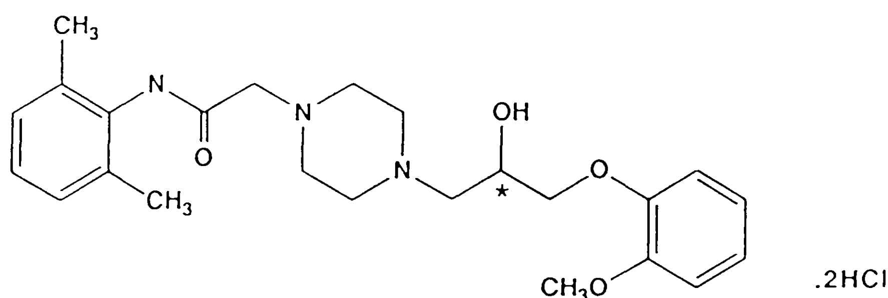
THE EFFECTS OF RANOLAZINE, A NOVEL ANTI-ISCHAEMIC AGENT, IN THE NORMOXIC AND ISCHAEMIC MYOCARDIUM

5.1 INTRODUCTION

Ranolazine, [(±)-N-(2,6-dimethyl-phenyl)4[2-hydroxy-3(2-methoxyphenoxy) propyl]-1-piperazine acetamide di-hydrochloride; RS-43285-193] is being developed by Syntex Research as a novel anti-anginal agent. It has been shown to be effective in inhibiting both the biochemical and gross electrocardiographical consequences of transient myocardial ischaemia in an intact dog model (Allely *et al.*, 1987; Allely and Alps, 1988) but without causing any direct haemodynamic effects. Ranolazine has been shown to be effective in preventing myocardial enzyme release in an intact primate model of ischaemia with reperfusion (Allely and Alps, 1990) and it has been suggested that ranolazine may alleviate the symptoms of transient myocardial ischaemia by altering substrate utilisation (Allely *et al.*, 1987). This compound has also been shown to reduce cAMP elevation in response to ischaemia in the *in vitro* perfused rat heart (Ferrandon *et al.*, 1992).

The studies described in this chapter have examined the effects of ranolazine on cAMP-mediated positive inotropism in comparison to other forms of inotropic intervention using the normoxic guinea-pig papillary muscle preparation. The effects of ranolazine have also been investigated in the guinea-pig low-flow ischaemic heart model to obtain direct biochemical evidence of cardioprotection and to gain some insight into the mechanism(s) underlying the anti-ischaemic properties of this compound.

RANOLAZINE



5.2 METHODS

5.2.1. The guinea-pig papillary muscle preparation

The papillary muscles were prepared as described in Section 2.3.2. All contractile force measurements were expressed as a % of the measurement made at the end of the 60 minutes equilibration period. At the end of the equilibration period, the following protocols were applied:

5.2.1.1. Ranolazine vs elevated extracellular calcium concentration $[Ca^{++}]_o$

Tissues were superfused for 20 minutes with either normal PSS (control experiments) or PSS containing 10μM ranolazine. This was followed by superfusion for 10 minutes with PSS containing 5mM Ca^{++} ± 10μM ranolazine.

5.2.1.2. Ranolazine vs Bay K 8644

Tissues were superfused for 20 minutes with either normal PSS or PSS containing 0.1, 1 or 10μM ranolazine. This was followed by superfusion for 60 minutes with PSS ± 1μM Bay K 8644. Ranolazine was present throughout this 60 minute period and control tissues were superfused with PSS + Bay K 8644 alone.

5.2.1.3. Ranolazine vs orciprenaline

In each experiment, 4 consecutive responses to 3μM orciprenaline were obtained. Each response was measured over a 5 minute period. The first orciprenaline response in each experiment was used as the control response and all subsequent responses were expressed as a % of that value. In control experiments,

each response to orciprenaline was followed by a wash-out period of 20 minutes. In experiments using ranolazine, each of these wash-out periods contained 0.1, 1 or 10μM ranolazine, respectively. Ranolazine was also added to the appropriate orciprenaline solutions.

5.2.1.4. Ranolazine vs forskolin

Two studies were carried out. The first examined the effects of ranolazine on the positive inotropic response to forskolin and the second was used to confirm the findings of the first study and in addition measure any changes to cAMP concentrations in the tissues.

In the first study, the effects of 20 minutes pre-treatment with a single concentration of either 0.01,0.1,1 or 10μM ranolazine followed by 30 minutes superfusion with 0.3μM forskolin was investigated. The relevant concentration of ranolazine was included in the forskolin solution and a time-matched control group perfused in a similar way but without ranolazine was included to assess the response to forskolin alone. Changes in contractile force were expressed as a % of the measurement made at the end of the equilibration period.

The response to forskolin in this preparation could be altered by the strength of the electrical stimulation. This may have been due to endogenous catecholamine release. In the second study, this effect was used to achieve a similar response to forskolin in each group of tissues. Thus, each group of tissues was perfused with 0.3μM forskolin for 20 minutes during which time the intensity of the electrical stimulation was adjusted to increase the contractile force to approximately 300% of the contractile force measured at the end of the equilibration period. This response was similar to that of control tissues in the previous study. The average stimulation voltage used during the equilibration period was 3.2V and this was increased to an average of 4.7V during the forskolin treatment period. Each group of tissues was then perfused with consecutive concentrations of ranolazine over the concentration

range 0.1, 1 and 10mcM. Each concentration was perfused for 20 minutes and forskolin was present in the ranolazine solutions. Control tissues were perfused with forskolin alone. Changes in contractile force were measured at the end of each 20 minute period. The tissues were frozen in liquid nitrogen at the end of the experiment. Any tissue which became arrhythmic or did not respond adequately to forskolin was excluded. An additional group of tissues perfused under equilibration conditions for the duration of the experiment were used to assess basal concentrations of cyclic AMP. The average voltage applied to these tissues was 3.3V.

5.2.2. The perfused guinea-pig heart preparation

The effects of 10μM ranolazine on the contractile activity and metabolic characteristics of the low-flow ischaemic heart were examined. Each heart was perfused as described in Section 2.3.1 and where appropriate was pre-treated with ranolazine for 20 minutes prior to ischaemia. The hearts were subjected to 30 minutes of low-flow ischaemia and when used, ranolazine was present throughout the ischaemic period. Untreated ischaemic hearts were perfused with normal PSS during the pre-treatment period. Normoxic control hearts were perfused with normal PSS throughout the experimental period. Contractile changes at the end of the pre-treatment period and during ischaemia were expressed as a % of the measurement made after 30 minutes equilibration. The hearts were frozen in liquid nitrogen after 30 minutes of ischaemia and biochemical analysis of ATP, creatine phosphate, glycogen and cyclic AMP was performed as described in Chapter 2. In addition the release of lactate and creatine kinase into the perfusate was measured.

5.3. RESULTS AND DISCUSSION

5.3.1. The effects of ranolazine in the guinea-pig papillary muscle preparation

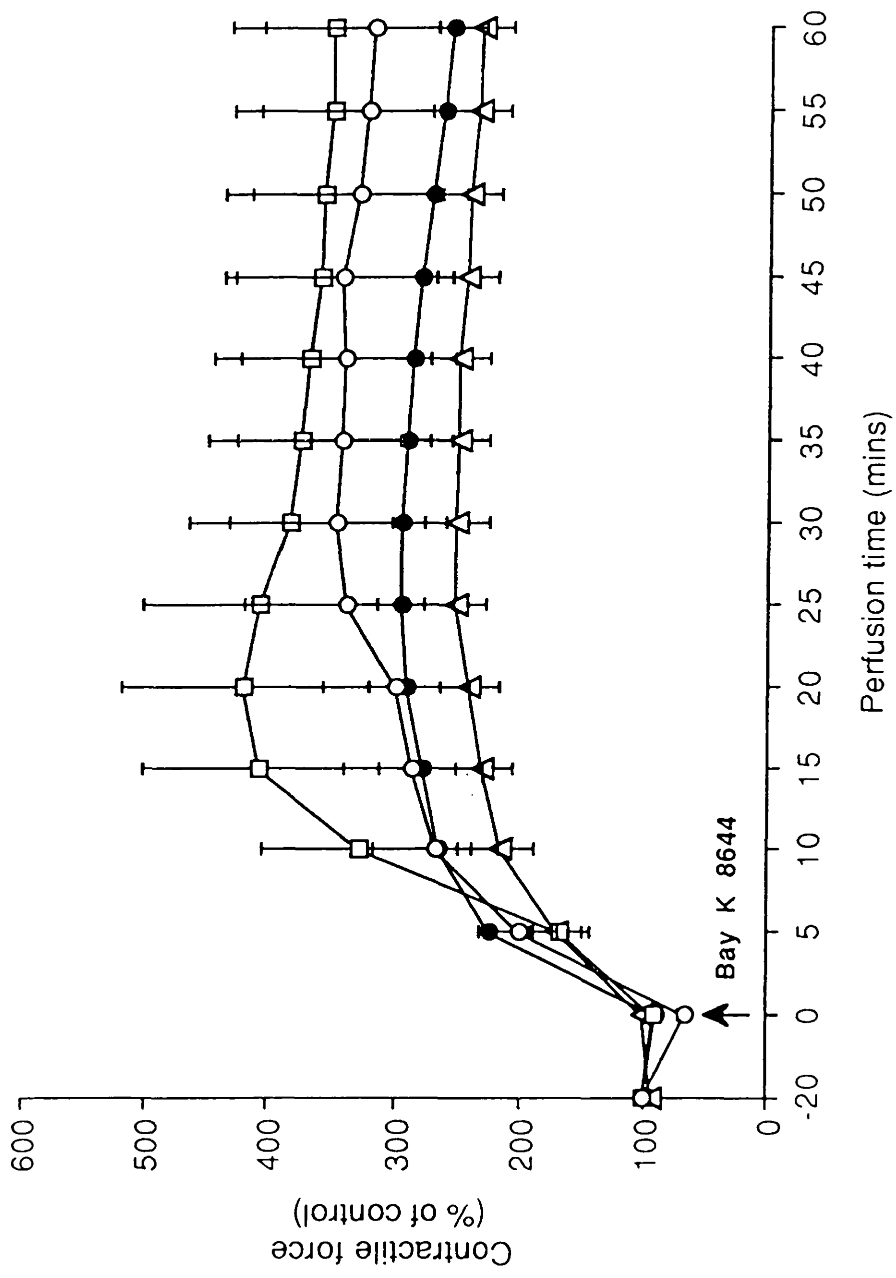
The effects of ranolazine on positive inotropic responses which were not mediated through changes in cAMP concentrations were investigated using elevated $[Ca^{++}]_o$ and the calcium entry facilitator Bay K 8644 (Schramm *et al.*, 1983). These results were compared with the effects of ranolazine on cAMP-mediated inotropic responses induced by the ß-adrenoceptor agonist orciprenaline and the direct activation of adenylate cyclase by forskolin (Seamon and Daly, 1983).

5.3.1.1. The effects of ranolazine on contractile force prior to the addition of elevated $[Ca^{++}]_o$ or Bay K 8644

Since each tissue was perfused with ranolazine for 20 minutes prior to the addition of elevated calcium or Bay K 8644, the effects of ranolazine during this pre-treatment period were measured. Time-matched control data obtained from experiments performed in the absence of ranolazine showed that under normoxic conditions, contractile force decreased to 88.1 ± 3.4% of control during the 20 minutes following the equilibration period. After treatment with 0.1, 1 or 10μM ranolazine for 20 minutes, contractile force decreased to 90.2 ± 1.8%, 92.3 ± 5.4% and 65.3 ± 5.2% ($p < 0.001$) of control, respectively. Only 10μM ranolazine had a significant effect on contractile force but this effect did not influence the subsequent responses to elevated $[Ca^{++}]_o$ or Bay K 8644.

Figure 5.1 The effect of ranolazine on the positive inotropic response to Bay K 8644 in the paced guinea-pig papillary muscle preparation

1μM Bay K 8644 was introduced after a 20 minute pre-treatment period with 0.1 (n= 6, open squares), 1 (n=5, open triangles) or 10μM (n=4, open circles) ranolazine. Ranolazine was also included in the Bay K 8644 solutions. Control tissues (n=5,filled circles) were perfused with normal PSS during the pre-treatment period. The values shown are mean ± S.E. and are expressed as a % of the measurement made at the end of a 60 minute equilibration period. The area under the curve for each treatment group was calculated and was compared to the control group by analysis of variance and application of Dunnett's t-test.



Contractile force
(% of control)
600
500
400
300
200
100
0
-20
0
5
10
15
20
25
30
35
40
45
50
55
60
Bay K 8644
Perfusion time (mins)

5.3.1.2. The effects of ranolazine on the positive inotropic response to elevated $[Ca^{++}]_o$ and Bay K 8644

In control experiments, increasing $[Ca^{++}]_o$ from 2mM to 5mM caused an increase in contractility to 471.2 ± 56.1% of control. After treatment with 10μM ranolazine for 20 minutes, the response to 5mM Ca^{++} was 472.3 ± 148.6% of control.

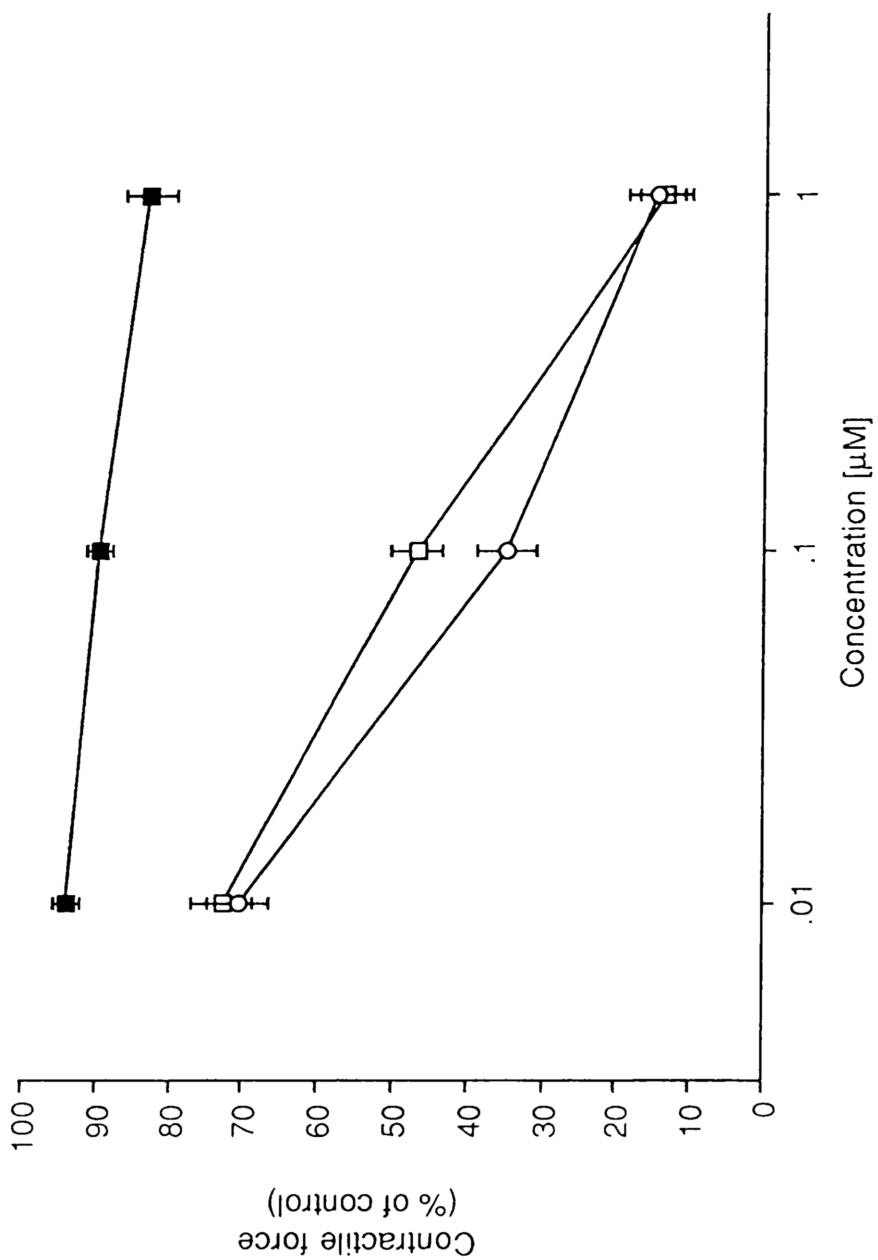
Superfusion with 1μM Bay K 8644 for 60 minutes increased contractile force to 256.6 ± 34.2% of control. Bay K 8644, in the presence of 0.1, 1 or 10μM ranolazine, increased contractile force to 350.7 ± 81.9%, 231.6 ± 23.2% and 318.8 ± 88.7% of control, respectively (Figure 5.1). Measurements of contractile force were made at 5 minute intervals throughout the 60 minute period and the area under the curve (AUC) for each treatment was compared with the control Bay K 8644 group. Ranolazine did not inhibit the positive inotropic responses associated with increased calcium influx in either experiment and it was concluded that the effect of 10μM ranolazine on contractile force during the pre-treatment period did not result in a subsequent inhibition of positive inotropism. The lack of inhibitory activity of ranolazine on the positive inotropic effects of elevated calcium and Bay K 8644 indicated that ranolazine does not interact directly with calcium entry through voltage-operated calcium channels. This is illustrated when the effects of ranolazine are compared to known calcium entry blockers (Figure 5.2).

5.3.1.3. The effects of ranolazine treatment prior to the addition of orciprenaline

The protocol used in these experiments is described in section 5.2.1.3. Data from control experiments without ranolazine treatment showed that during the wash periods following each of the 4 orciprenaline responses, contractile force decreased to 88.4 ± 6.6%, 72.9 ± 5.5% and 65.7 ± 5.1% of control, respectively. When ranolazine was included in the consecutive wash periods, contractile force

Figure 5.2 The comparative effects of ranolazine, nicardipine and nifedipine on contractile activity in the paced guinea-pig papillary muscle preparation

The effects of 0.01, 1 and 10μM ranolazine (n=6, filled squares) on contractile force were compared with nicardipine (n=6, open squares) and nifedipine (n=6, open circles). Each increasing concentration was perfused for 30 minutes and changes to contractile force at the end of this period were expressed as a % of the measurement made at the end of the 60 minute equilibration period.



Contractile force
(% of control)
100
90
80
70
60
50
40
30
20
10
0
.01
.1
1
Concentration [µM]

decreased to 86.8 ± 7.5% (0.1μM), 68.0 ± 5.2% (1μM) and 46.9 ± 3.8% (10μM), respectively. Compared to the corresponding control values, 10μM ranolazine had a small but significant negative inotropic effect ($p < 0.05$) on contractile force during the pre-treatment period. This was similar to the results described in section 5.3.1.1.

5.3.1.4. The effects of ranolazine on the positive inotropic response to orciprenaline

In the control experiments, the 4 consecutive responses to orciprenaline were 406.5 ± 62.0%, 379.1 ± 67.2%, 322.1 ± 57.6% and 294.5 ± 55.1% of control (Figure 5.3a). This reduction of the orciprenaline responses would be expected on repeated exposure to a ß-adrenoceptor agonist due to desensitisation of the tissue.

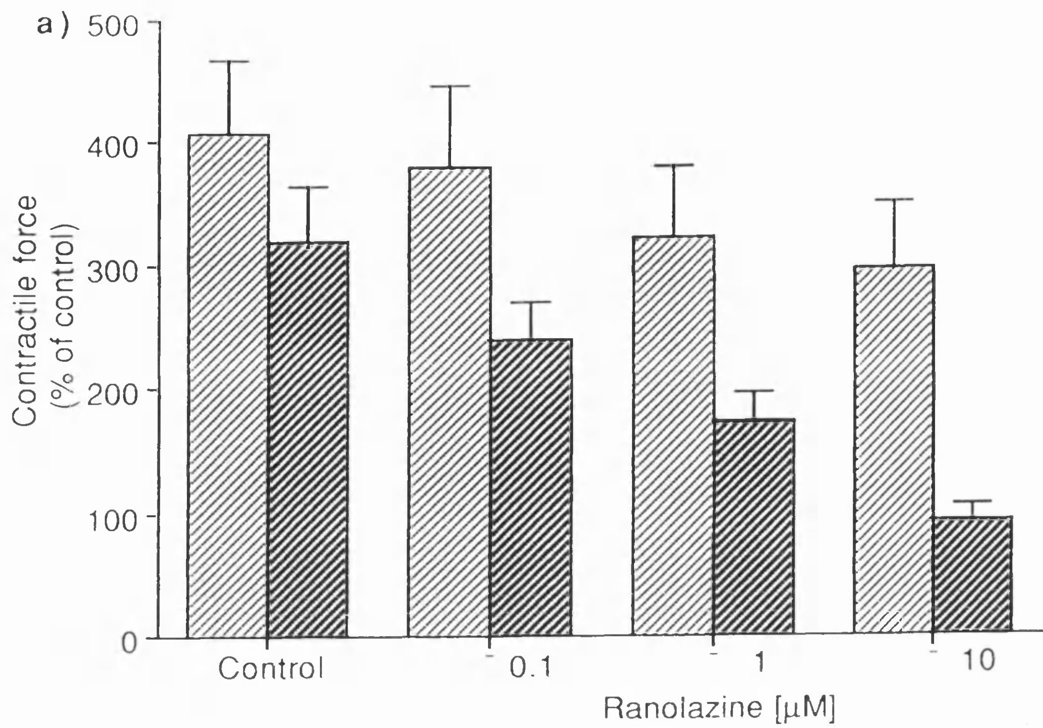
Using the same protocol, the effects of ranolazine on consecutive orciprenaline responses were studied in a separate series of experiments. In these experiments, the first response to orciprenaline was only 319.1 ± 44.2% of control (Figure 5.3a) but this was not significantly different from the first response to orciprenaline in the control experiments described in the previous paragraph. Ranolazine was included in the perfusate after this response had been obtained and the subsequent responses to orciprenaline in the presence of ranolazine were 239.2 ± 30.9% (0.1μM), 171.8 ± 23.8% (1μM) and 92.3 ± 12.5% (10μM).

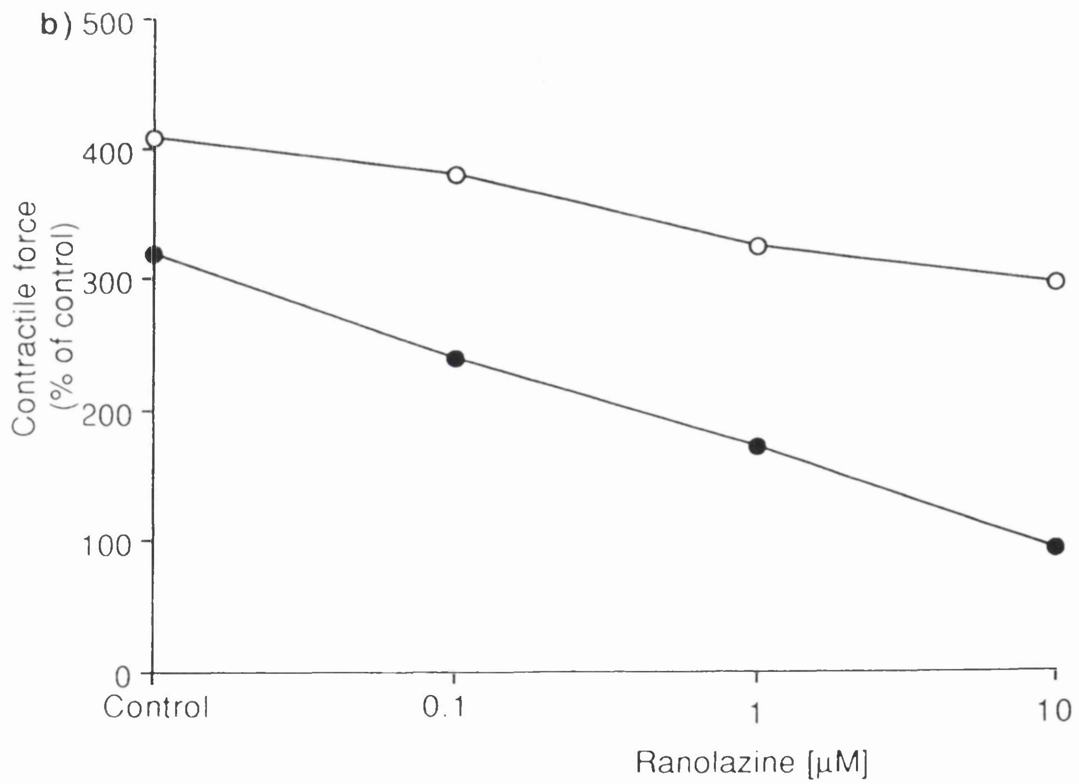
The regression co-efficients of the responses to orciprenaline with and without ranolazine present were compared (Figure 5.3b) and statistical analysis showed that the regression slopes were significantly different ($p < 0.01$). The regression analysis showed that although the consecutive responses to orciprenaline decreased in the control experiments, this decrease was significantly greater when orciprenaline was used in the presence of ranolazine.

The effect of ranolazine on the response to orciprenaline suggested an interaction with cardiac ß-adrenoceptors. The binding affinity (pK_i) of ranolazine at

Figure 5.3 The effect of ranolazine on the positive inotropic response to orciprenaline in the paced guinea-pig papillary muscle preparation

The data shown are comparisons of repeated responses to orciprenaline (see section 5.2.3) with (n=16, dark hatch bars) and, in a separate group of tissues, without (n=8, light hatch bars) ranolazine (Figure a). The values are mean ± S.E. expressed as a % of the measurement made at the end of the 60 minute equilibration period. Figure b) shows a regression analysis of this data. The regression slope calculated for orciprenaline in the presence of ranolazine (closed circles) was significantly greater ($p < 0.01$) than the control group (open circles).


a)
500
400
300
200
100
0
Contractile force
(% of control)
Control
0.1
1
10
Ranolazine [μM]


b)
500
400
300
200
100
0
Contractile force
(% of control)
Control
0.1
1
10
Ranolazine [μM]

rat cardiac β_1-adrenoceptors is 5.1 (unpublished data of C.M.Brown, Syntex Research Scotland), however, Ferrandon *et al.* (1992) showed that ranolazine inhibited isoprenaline-induced cAMP elevation in rat heart, in the nanomolar concentration range. Therefore, it was unclear whether the inhibitory effects of ranolazine observed in the guinea-pig papillary muscle preparation, were due to a direct effect on the ß-adrenoceptor or the result of an indirect intracellular action involving cAMP. The latter possibility was investigated further using forskolin to elevate the cAMP concentration.

5.3.1.5. The effects of ranolazine on the positive inotropic response to forskolin

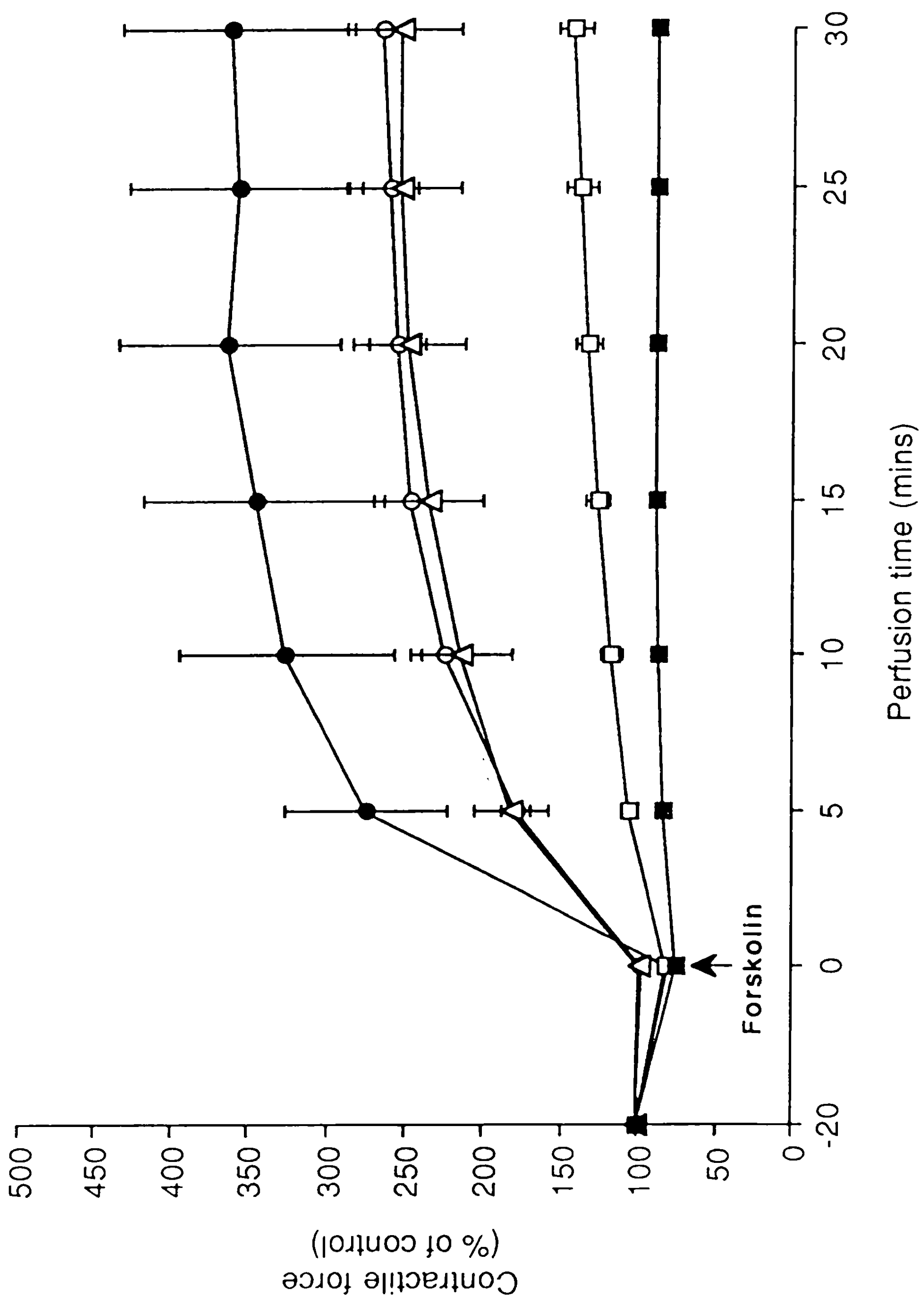
Compared to the time-matched control group, ranolazine had no significant inhibitory effects on contractile force during the 20 minute pre-treatment period. In the control group, contractile force decreased to 80.6 ± 4.5% of control during this period and the values for 0.01, 0.1, 1 and 10μM ranolazine were 97.0 ± 5.4%, 97.9 ± 3.8%, 82.1 ± 1.9% and 74.9 ± 2.9% of control, respectively (Figure 5.4).

Superfusion with forskolin for 30 minutes increased contractile force to 361.8 ± 72.5% of control. Ranolazine inhibited this response in a concentration-dependent manner and the respective values for 0.01, 0.1, 1 and 10μM ranolazine after 30 minutes were 265.4 ± 18.7%, 252.6 ± 36.7%, 142.1 ± 10.5% and 88.5 ± 5.0% of control (Figure 5.4). The time-course of the response to forskolin and the effects of ranolazine were statistically analysed by measuring the area under the curves and this showed that the inhibitory effects of 1 and 10μM ranolazine were significant ($p < 0.01$).

Since these tissues were electrically-paced, one possible explanation for the positive inotropic response to forskolin in these experiments was that ß-adrenoceptor activation by release of endogenous catecholamines also contributed to cAMP elevation. Thus, ranolazine could, by affecting ß-adrenoceptor activation, reduce cAMP elevation in the presence of forskolin under these conditions.

Figure 5.4 The effects of ranolazine on the positive inotropic response to forskolin in the paced guinea-pig papillary muscle preparation

0.3μM forskolin was introduced after a 20 minute pre-treatment period with 0.01 (n=12, open circles), 0.1 (n=13, open triangles), 1 (n=26, open squares) or 10μM (n=18, closed squares) ranolazine. Ranolazine was also included in the forskolin solutions. Control tissues (n=14, filled circles) were perfused with normal PSS during the pre-treatment period. The values shown are mean ± S.E. and are expressed as a % of the measurement made at the end of the 60 minute equilibration period. The area under the curve for each treatment group was calculated and was compared to the control group by analysis of variance and application of Dunnett's t-test.



Contractile force
(% of control)
500
450
400
350
300
250
200
150
100
50
0
-20
0
5
10
15
20
25
30
Perfusion time (mins)
Forskolin

Therefore, a second similar study was carried out in which the cAMP concentration in the tissues was measured at the end of the treatment periods.

5.3.1.6. The effects of ranolazine on cAMP and the positive inotropic response to forskolin

Two groups of tissues were used in this study and a control response to forskolin was obtained at the start of each experiment, forskolin increased contractile force to 317.9 ± 11.4% and 301.7 ± 24.0% of control in both groups, respectively. In the latter group, subsequent treatment with ranolazine decreased contractile force to 270.8 ± 18.3% (0.1μM), 229.5 ± 18.5% (1μM) and 130.9 ± 18.7% (10μM) of control. The corresponding values in the untreated forskolin control group were 281.0 ± 19.7%, 286.8 ± 23.0% and 245.3 ± 22.1% of control (Figure 5.5a).

Measurements of the tissue concentration of cAMP showed that basal levels in a group of tissues stimulated at 3.3V and superfused with normal PSS for the duration of these experiments were 4.8 ± 0.5pmol/mg protein (n=12). In the untreated forskolin control group (n=12), the cAMP concentration was 14.1 ± 1.4pmols/mg protein compared to 16.1 ± 3.0pmol/mg protein in the ranolazine-treated forskolin group (n=8). Statistical analysis showed that compared to the basal concentration, cAMP was significantly higher in both the forskolin control and ranolazine-treated forskolin groups, however ranolazine did not reduce cAMP in the presence of forskolin (Figure 5.5b).

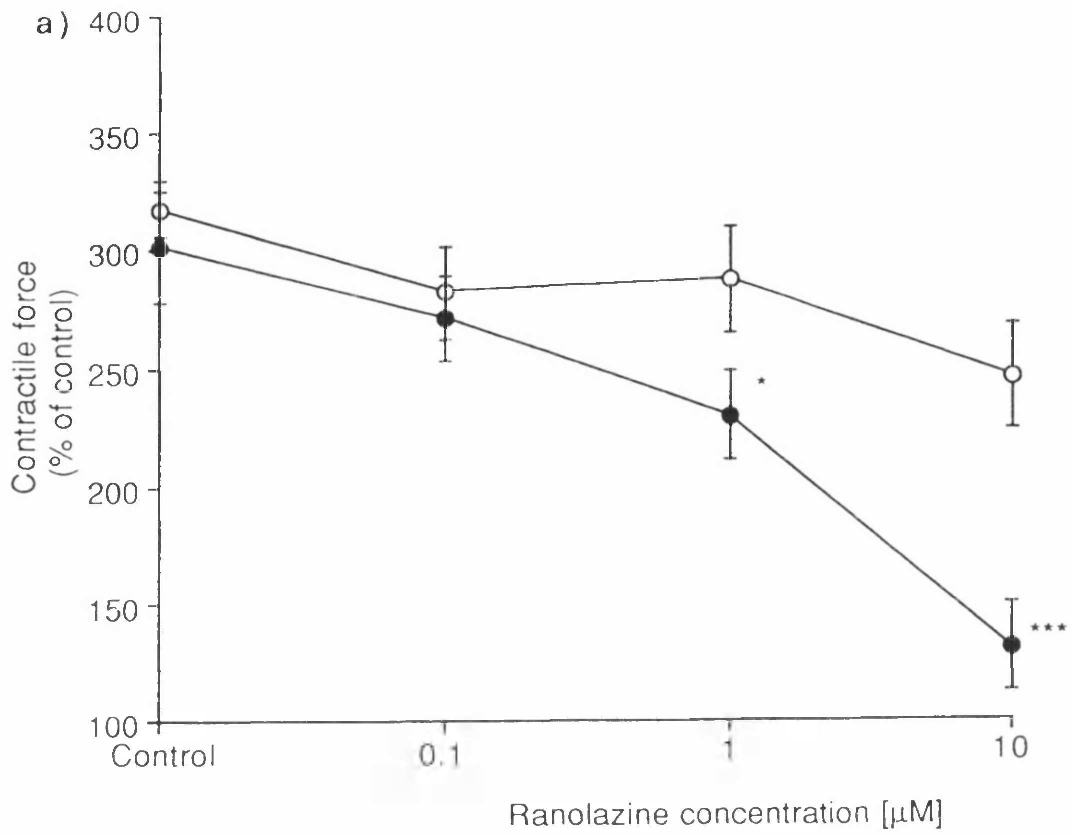
The levels of cAMP determined in this study indicated that the protocol used to increase contractile force in the presence of forskolin was associated with an increase in cAMP concentrations. However the decrease in contractile force observed in the presence of ranolazine was not associated with a decrease in cAMP.

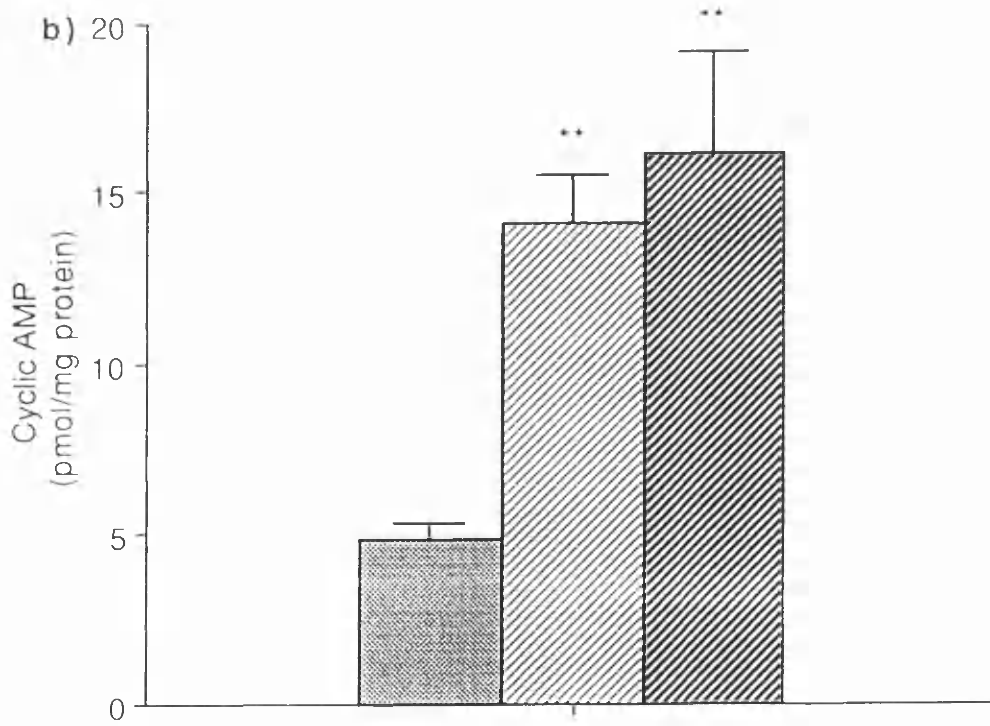
The ß-adrenoceptor binding affinity of ranolazine is low in comparison to known ß-adrenoceptor blockers (e.g. propanolol pK_i 7.9) and one possible

Figure 5.5 The effects of ranolazine on cyclic AMP and the positive inotropic response to forskolin in the paced guinea-pig papillary muscle preparation

The effects of ranolazine (n=10, closed circles) on the contractile response to 0.3μM forskolin (Figure a) were compared with control tissues perfused with forskolin alone (n=12, open circles). The values shown in Figure a) are mean ± S.E. expressed as a % of the measurement made at the end of the 60 minutes equilibration period prior to the addition of forskolin. Each concentration of ranolazine was perfused for 20 minutes and changes to contractile force were measured at the end of this period. Forskolin was present throughout the ranolazine treatment period. Statistical analysis comparing the inotropic effects of forskolin with and without ranolazine treatment was by analysis of variance and application of a 2-sided t-test (*$p < 0.05$; ***$p < 0.001$).

The concentration of cyclic AMP in tissues sampled at the end of these experiments after treatment with forskolin alone (light hatch bars) or forskolin + ranolazine (dark hatch bars) was compared to that of control tissues (shaded bars) perfused with normal PSS over the same experimental duration. Statistical comparison between the control group and the treated groups was by analysis of variance and application of Dunnett's t-test (**$p < 0.01$).


a)
400
350
300
250
200
150
100
Contractile force
(% of control)
*
***
Control
0.1
1
10
Ranolazine concentration [μM]


b)
20
15
10
5
0
Cyclic AMP
(pmol/mg protein)
**
**

interpretation of these results is that ranolazine interacts with a post-cAMP phosphorylation site (e.g. cAMP-dependent protein kinase). However, the observed effects of ranolazine may be the result of a more complex interaction. Recent evidence has suggested that the elevation of cAMP by forskolin and ß-adrenoceptor stimulation may involve separate intracellular compartments (Worthington and Opie, 1992).

5.3.2 The effects of ranolazine in the paced low-flow ischaemic perfused heart

Ranolazine exerted pronounced effects on contractile force when cAMP was elevated under normoxic conditions. Hoever, since no evidence for increased cAMP had been found in the guinea-pig low-flow ischaemic heart, it was of interest to study the anti-ischaemic effects of ranolazine in this model. The effects of a concentration (10μM) which had been shown to give maximal protection against several indices of ischaemic damage in in vitro rat heart preparations (Ferrandon *et al.*, 1988; 1990) were investigated.

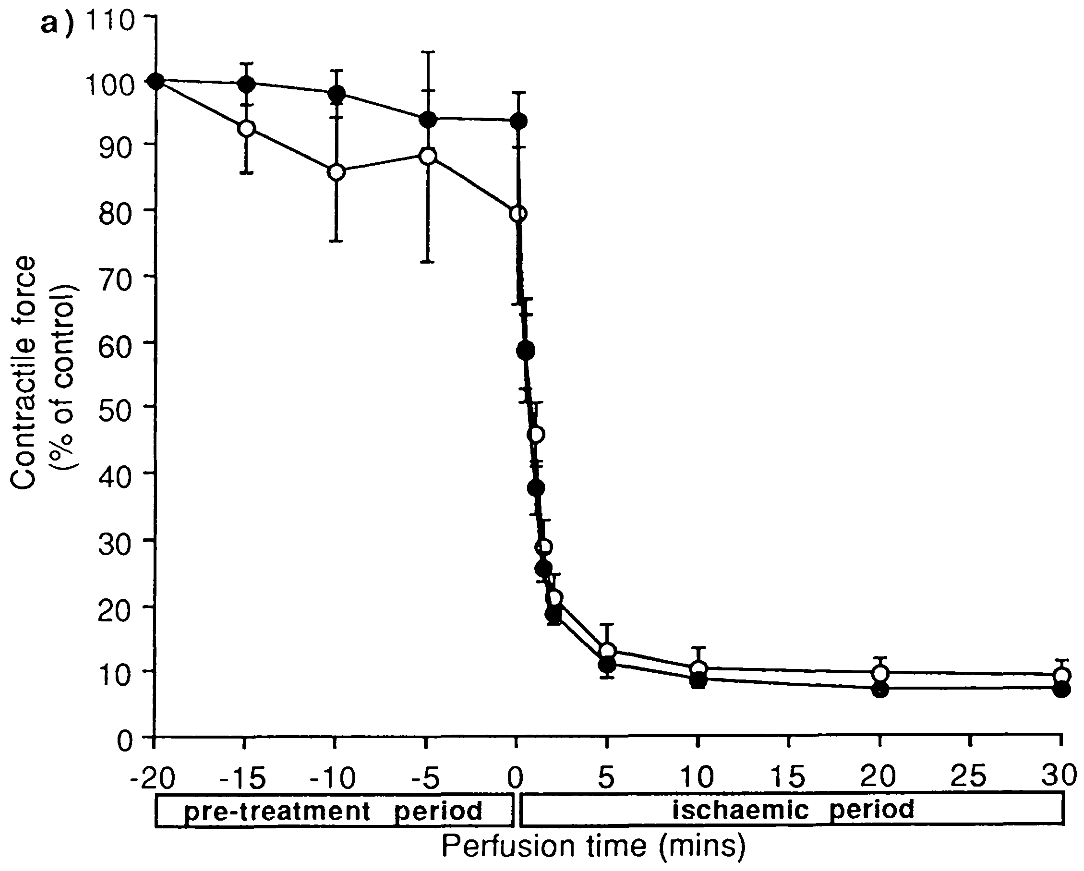
5.3.2.1 The effects of ranolazine on contractile activity during pre-treatment and low-flow ischaemia

The effects of ranolazine during the 20 minute pre-treatment period and during ischaemia were compared to control measurements from ischaemic hearts perfused in the absence of ranolazine (Figures 5.6a and 5.6b). At the end of the pre-treatment period, contractile force in the control group was 79.1 ± 13.4% of control compared to 93.5 ± 4.3% after ranolazine treatment. Diastolic tension in the two groups was 96.1 ± 4.6% and 99.2 ± 2.5% of control, respectively. Therefore 10μM ranolazine did not affect contractile function prior to ischaemia.

In the first 5 minutes of ischaemia, contractile force decreased rapidly to 12.9 ±

Figure 5.6 The effects of ranolazine on contractile function during pre-treatment and low-flow ischaemia in the paced perfused guinea-pig heart

After 20 minutes pre-treatment with 10μM ranolazine, each heart was perfused under low-flow ischaemic conditions for 30 minutes (n=6, closed circles). Ranolazine was also present during the ischaemic period. Control hearts (n=5, open circles) were perfused with normal PSS during the pre-treatment period. Changes to contractile force (Figure a) and diastolic tension (Figure b) were expressed as a % of the measurement made at the end of the equilibration period prior to the addition of ranolazine and the values shown are mean ± S.E.


a)
110
100
90
80
70
60
50
40
30
20
10
0
Contractile force
(% of control)
-20
-15
-10
-5
0
5
10
15
20
25
30
pre-treatment period
ischaemic period
Perfusion time (mins)

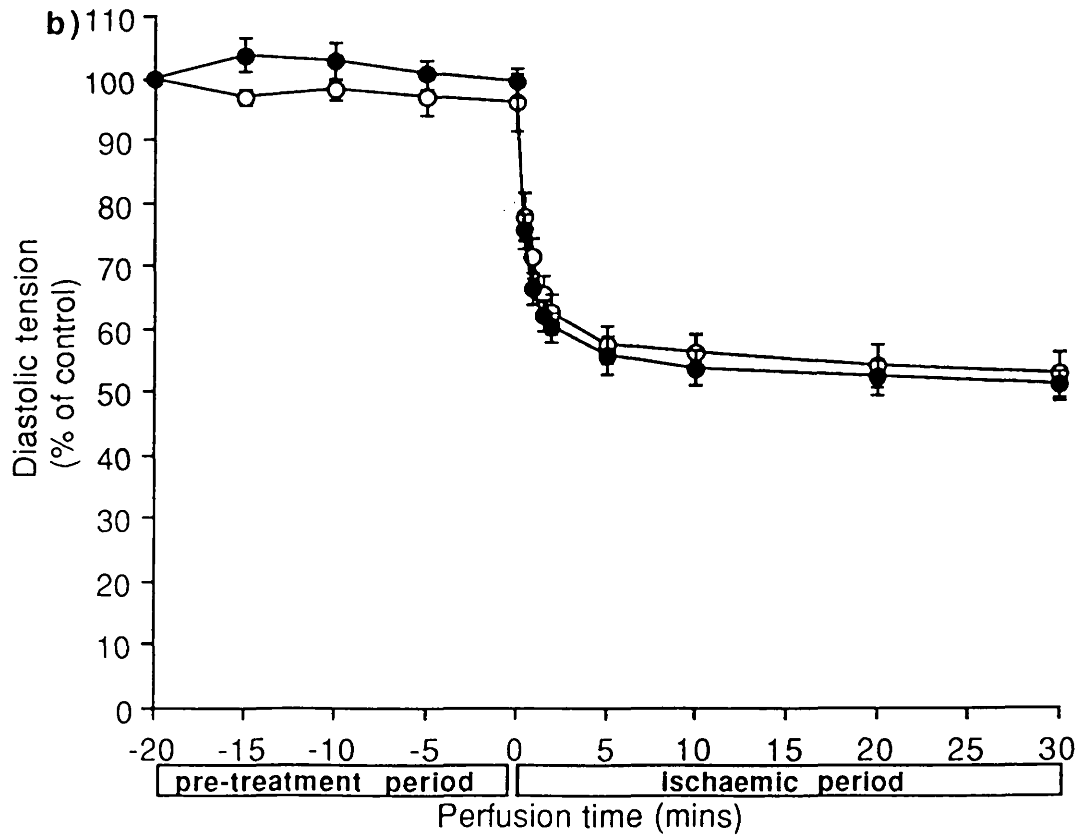

b)
110
100
90
80
70
60
50
40
30
20
10
0
Diastolic tension
(% of control)
-20
-15
-10
-5
0
5
10
15
20
25
30
pre-treatment period
ischaemic period
Perfusion time (mins)

Table 5.1 The effects of ranolazine on the metabolic characteristics of paced low-flow ischaemic perfused guinea-pig hearts

Experimental conditions and treatments were as described in the legend for Figure 5.6. A group of time-matched normoxic hearts was included for comparison with the ischaemic hearts. The values shown are mean ± S.E. Cyclic AMP values are expressed as nmol/gm dry wt and all other values are μmol/gm dry wt. Statistical comparison to the corresponding normoxic values was by analysis of variance and application of Dunnett's t-test (**$p < 0.01$). An additional comparison between the ranolazine-treated group and ischaemia alone was by unpaired t-test (#$p < 0.05$).

Experimental	**Tissue content**				
Condition	ATP	Creatine phosphate	Glycogen	Lactate	cAMP
Normoxia (n=5)	11.8 ± 0.8	10.4 ± 0.1	92.9 ± 10.9	4.7 ± 0.6	1.4 ± 0.2
Low-flow ischaemia (n=5)	5.6 ± 0.8 **	1.9 ± 0.3 **	38.9 ± 5.0 **	42.9 ± 2.2 **	1.4 ± 0.1
Low-flow ischaemia + ranolazine (n=6)	9.0 ± 1.1 #	2.3 ± 0.3 **	43.0 ± 3.0 **	38.5 ± 3.5 **	1.2 ± 0.1

4.0% in the control group and 10.8 ± 0.6% of control in the ranolazine treated group. After this time, contractile force remained similar in both groups and was 8.8 ± 2.4% and 6.7 ± 0.5% of control, respectively, at the end of the ischaemic period. Diastolic tension in the two groups was reduced to 52.6 ± 3.6% and 50.9 ± 2.4% of control, respectively, after 30 minutes of ischaemia and these values were not significantly different.

5.3.2.2 The effect of ranolazine on the metabolic characteristics of the low-flow ischaemic heart

The effects of ischaemia with and without 10μM ranolazine on ATP, creatine phosphate, glycogen and cAMP contents were compared to values obtained from normoxic hearts perfused over the same time-period (Table 5.1.)

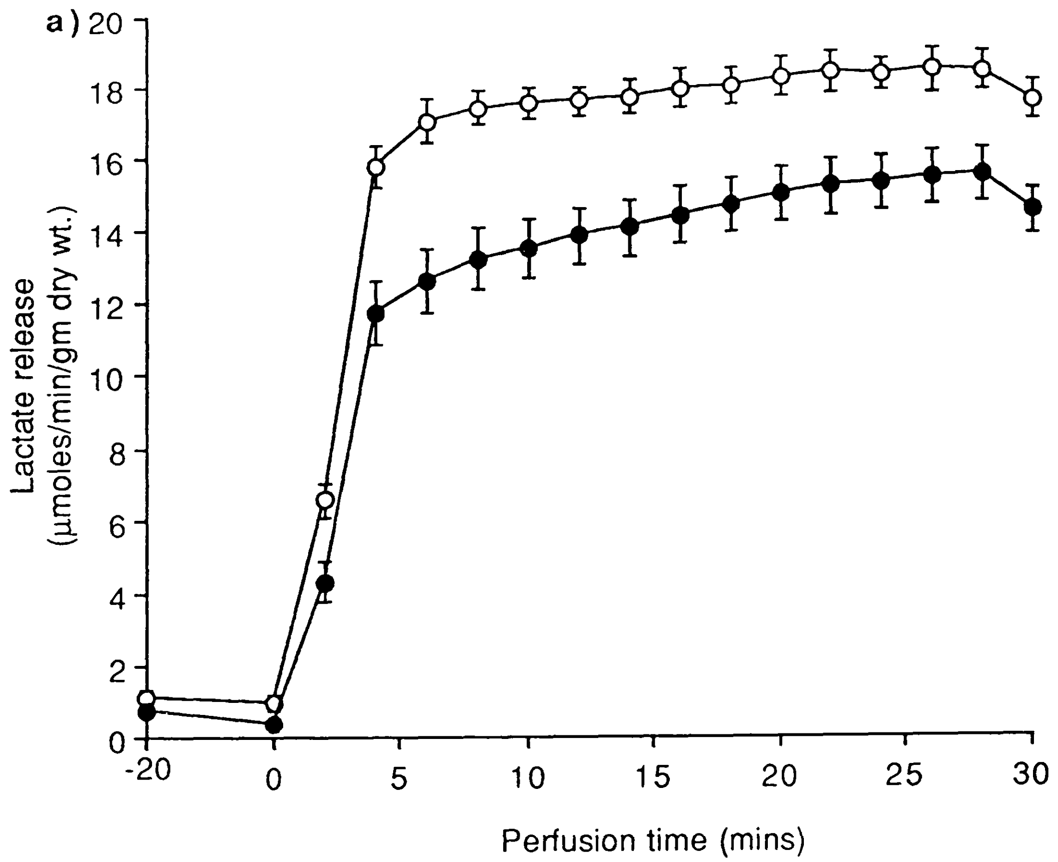
During low-flow ischaemia there was a reduction of ATP and creatine phosphate to approximately 50% and 18% of the normoxic values, respectively. Similarly, glycogen was reduced to approximately 40%. However, at the end of the ischaemic period in the ranolazine-treated group, ATP was approximately 75% of the normoxic value which was significantly higher than after ischaemia alone. Ranolazine did not affect the amount of creatine phosphate and glycogen present at the end of the ischaemic period. As in previous low-flow ischaemic experiments, no changes to cAMP concentrations were found in the ischaemic hearts.

Although tissue lactate concentrations in both ischaemic groups were not significantly different (Table 5.1), the concentration of lactate in the perfusate sampled from the ranolazine group was significantly lower than that of ischaemia alone (Figure 5.7a). A similar reduction of creatine kinase release was also found in the ranolazine group (Figure 5.7b).

The preservation of ATP and the reduction of lactate and creatine kinase release was not associated with changes in cAMP concentrations in the ischaemic hearts. This suggested that, in this model, the anti-ischaemic effects of ranolazine were

Figure 5.7 The effects of ranolazine on lactate and creatine kinase release during low-flow ischaemia in the paced perfused guinea-pig heart

Experimental conditions and treatments were as described for Figure 5.6. Lactate release into the perfusate with (n=6, closed circles) and without ranolazine (n=5, open circles) is shown in Figure a) and creatine kinase release with (n=4, closed circles) and without ranolazine (n=6, open circles) are shown in Figure b). The values shown are mean ± S.E. and statistical analysis comparing the control and treated groups was by analysis of variance and application of a 2-sided t-test. Statistical significance ($p < 0.05$) was found at all points except where indicated.


a)
Lactate release
(µmoles/min/gm dry wt.)
0
2
4
6
8
10
12
14
16
18
20
-20
0
5
10
15
20
25
30
Perfusion time (mins)

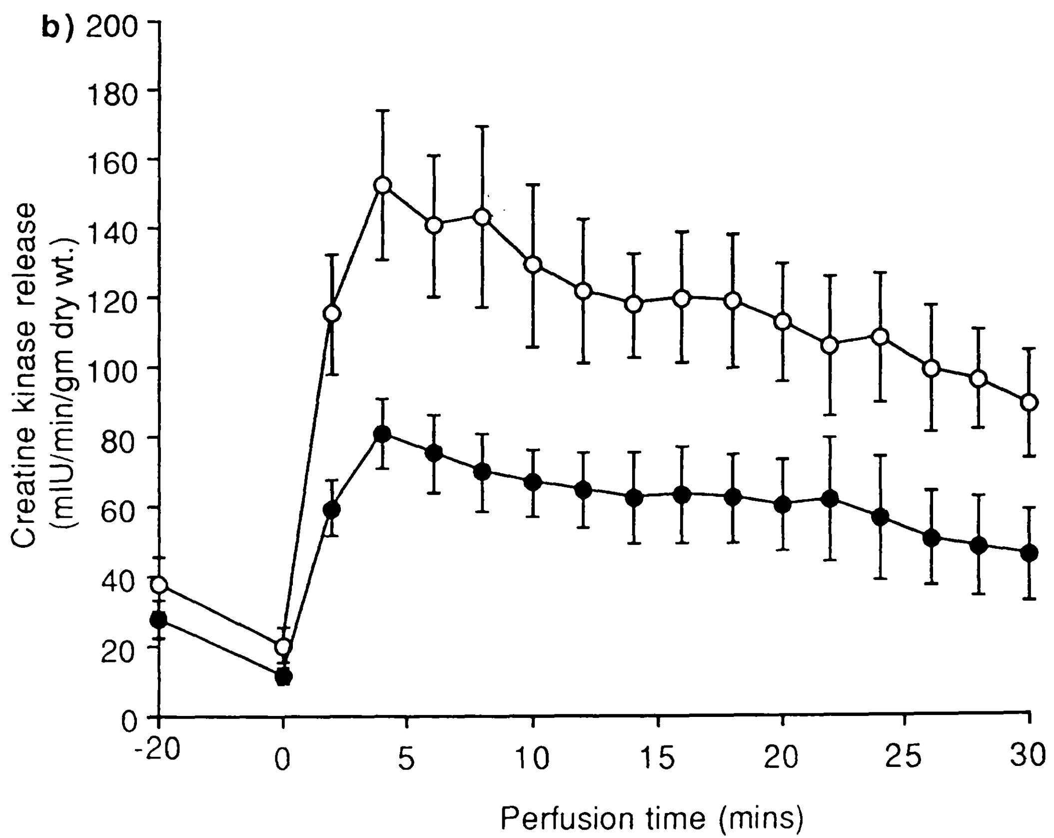
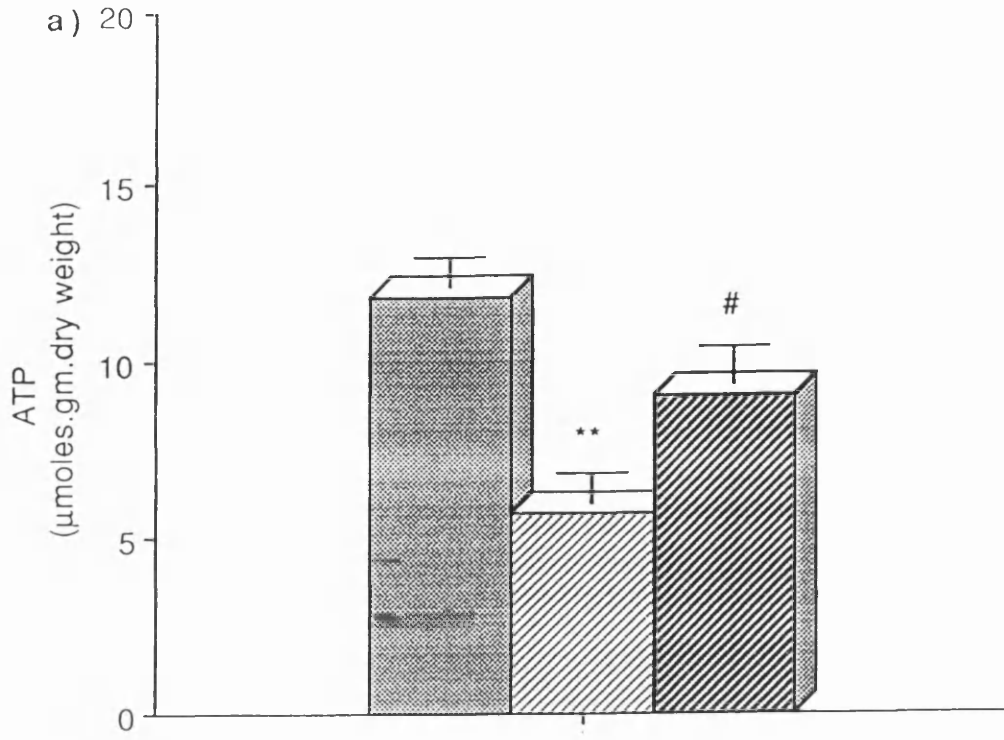
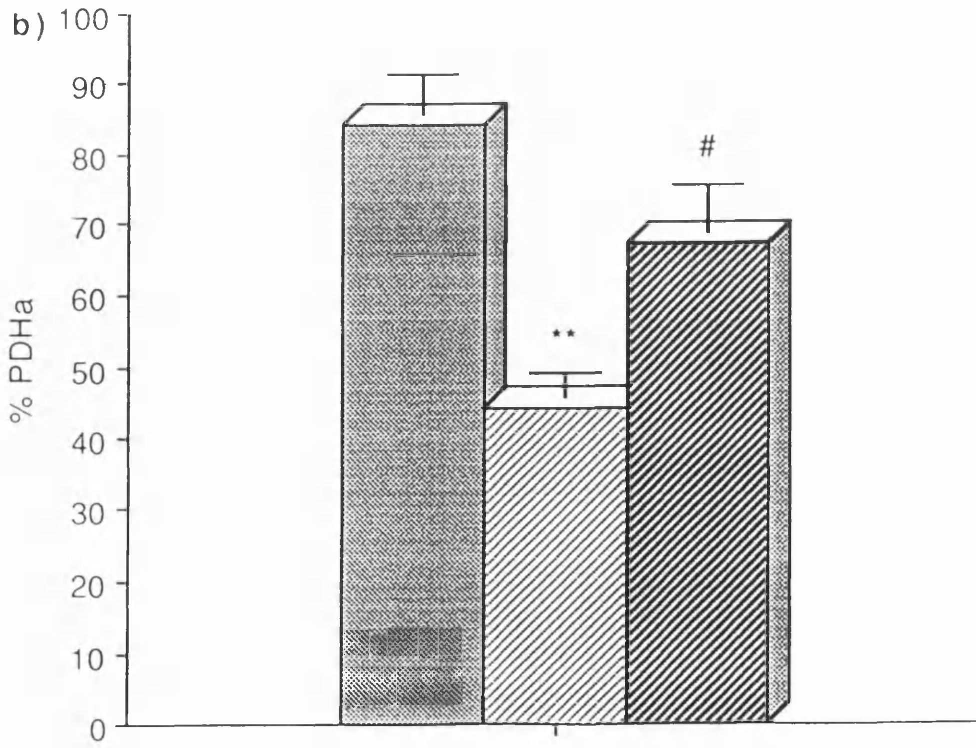

b)
Creatine kinase release
(mIU/min/gm dry wt.)
0
20
40
60
80
100
120
140
160
180
200
-20
0
5
10
15
20
25
30
Perfusion time (mins)

Figure 5.8 The comparative effects of ranolazine on ATP and PDHa following low-flow ischaemia in the paced perfused guinea-pig heart

The tissue content of ATP (Figure a) and PDHa (Figure b) were measured in time-matched normoxic (n=5, shaded bars), low-flow ischaemic (n=5, light hatch bars) and low-flow ischaemic + 10μM ranolazine treated hearts (n=6, dark hatch bars). Ranolazine was perfused for 20 minutes prior to the 30 minute ischaemic period. The values shown are mean ± S.E. and statistical comparison of the ischaemic groups and normoxia was by analysis of variance and application of Dunnett's t-test (**$p < 0.01$). An additional comparison between the ranolazine-treated group and ischaemia alone was by unpaired t-test (#$p < 0.05$).



a)
20
15
10
5
0
ATP
(µmoles.gm.dry weight)
**
#



b)
100
90
80
70
60
50
40
30
20
10
0
% PDHa
**
#

independent of cAMP-mediated events. At this stage, changes to the activity of pyruvate dehydrogenase in the ischaemic tissues were considered as a possible explanation for the effects of ranolazine in these experiments.

The level of activated pyruvate dehydrogenase (PDHa) measured in normoxic perfused control hearts was 84.2 ± 5.5% of the total PDH content of the tissues (Figure 5.8). At the end of the low-flow ischaemic period, PDHa was significantly reduced to 44.2 ± 3.2% of the total PDH content. The amount of PDHa measured at the end of ischaemia in the ranolazine treated group was 67.2 ± 6.8% of the total PDH content and was not significantly different from the normoxic value.

5.4 DISCUSSION

This study has shown that ranolazine can prevent some of the deleterious consequences of ischaemia in this in vitro model and is supportive of the reports of the cardioprotective effects seen in vivo and in vitro. The preservation of ATP may contribute to the improvement of functional parameters on reperfusion following ischaemia observed in some earlier studies.

PDH occupies a key site in metabolic substrate selection and is subject to complex regulation (Randle, 1986). The values obtained for total PDH activity in this study are similar to those found previously (Bunger *et al.*, 1983) but the control values for PDHa are high as a % of total activity (McCormack and Denton, 1989). However, they are similar to those previously found in glucose-perfused guinea-pig hearts (Hansford *et al.*, 1990). In rat heart, ischaemia has been shown to lead to inactivation of PDH (Kobayashi and Neely, 1983; Patel and Olson, 1984) and the present study shows that a similar situation may occur in the guinea-pig.

The anti-ischaemic effects of ranolazine appear to differ from that of many commonly prescribed anti-anginal agents (e.g. ß-adrenoceptor and calcium channel blocking agents) since general haemodynamics are unaffected (Allely and Alps, 1988; 1989). Although it is not clear whether activation of PDH or prevention of

the ischaemic inhibition of this enzyme is involved in the cardioprotective action of ranolazine, such a mechanism could account for the reduction of lactate release and preservation of the ATP concentration by allowing more lactate to be used as a substrate. Since carbohydrate metabolism utilises less oxygen, this effect would be most beneficial under conditions of limited oxygen supply such as reduced coronary flow. Tissue ATP preservation and perhaps reduced lactic acidosis may thus allow increased preservation of cellular viability and hence account for the observed effects on CK release as an indicator of ischaemic cell damage.

The present observations offer some insights into the potential mechanisms for the cardio-protective and anti-anginal effects of ranolazine but it will be important to determine whether these effects are due to a direct action on PDH or are the consequence of an indirect metabolic action elsewhere.

CHAPTER SIX

THE INVOLVEMENT OF K_{ATP}-DEPENDENT CHANNEL ACTIVATION IN EARLY CONTRACTILE FAILURE DURING CARDIAC ISCHAEMIA AND HYPOXIA *IN VITRO*

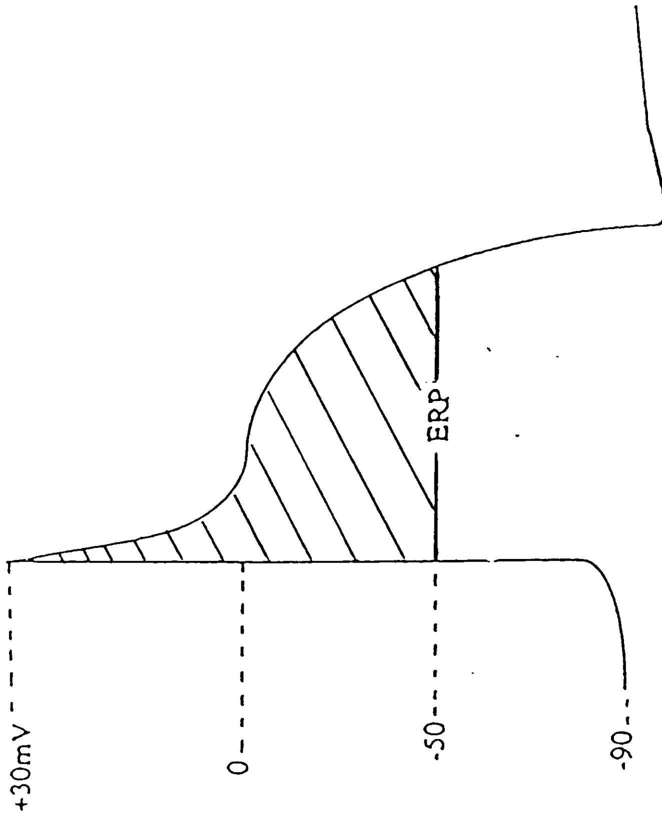
6.1 INTRODUCTION

Early contractile failure (ECF) is a well recognised phenomenon in oxygen-deprived tissue under both ischaemic and hypoxic conditions, however, the specific mechanism underlying this effect has not been identified (Allen and Orchard, 1983, 1987). Although several mechanisms have been considered including acidosis and effects on the contractile process, depletion of ATP and the subsequent opening of ATP - dependent K^+ channels (K_{ATP}), identified by Noma, (1983), may be of importance in this rapid loss of contractile activity in ischaemic and hypoxic myocardium. During ischaemia and hypoxia, the activation of K_{ATP}-channels has been associated with shortening of the action potential duration (Nichols *et al.*, 1991) and loss of intracellular K^+ (Vleugels *et al.*, 1980) but whether these events are linked remains unclear (Askenes, 1992).

This chapter investigates the ECF reponse to ischaemia and hypoxia in the guinea-pig perfused heart model developed in this thesis and examines the effects of K_{ATP}-channel activation using cromakalim (Escande *et al.*, 1988) and K_{ATP}-channel blockade using glibenclamide (Kantor et al., 1990) under normoxic and hypoxic conditions in the guinea-pig papillary muscle preparation.

Figure 6.1 The cardiac action potential and related transmembranal ionic changes

Figure a) shows the stages of depolarisation and repolarisation of the action potential during normal pacemaker activity. Depolarisation (0) is associated with a rapid influx of Na^+ and a resultant change in membrane potential from around -90mV to +30mV (1). This is followed by the influx of Ca^{++} (2) and the efflux of K^+ (3) which contribute to the repolarisation phase. When complete, the next depolarisation phase commences (4). The duration of the absolute or effective refractory period (ERP, for definition see text) is shown in Figure b).


b)
+30mV
0
-50
-90
ERP

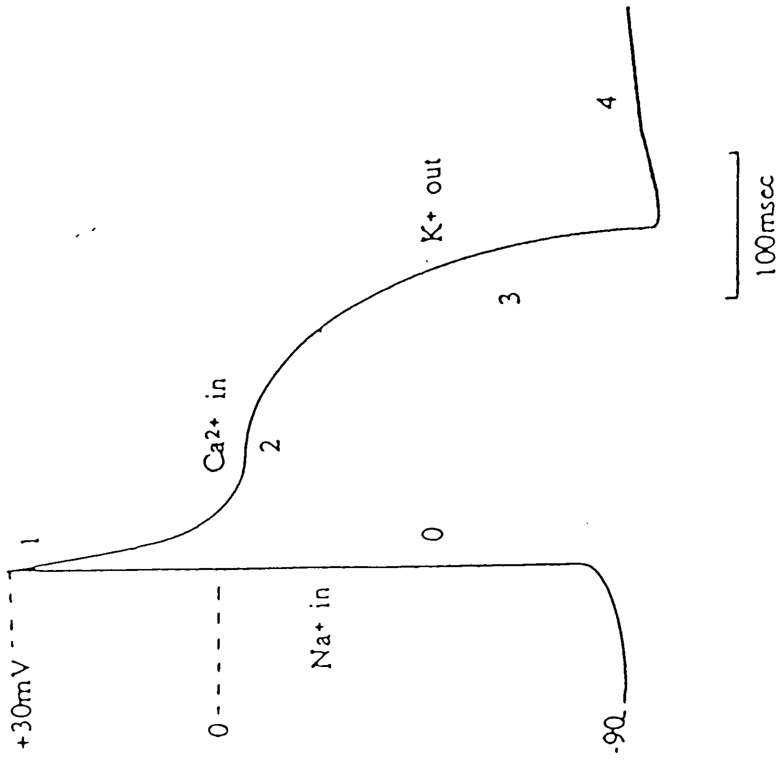
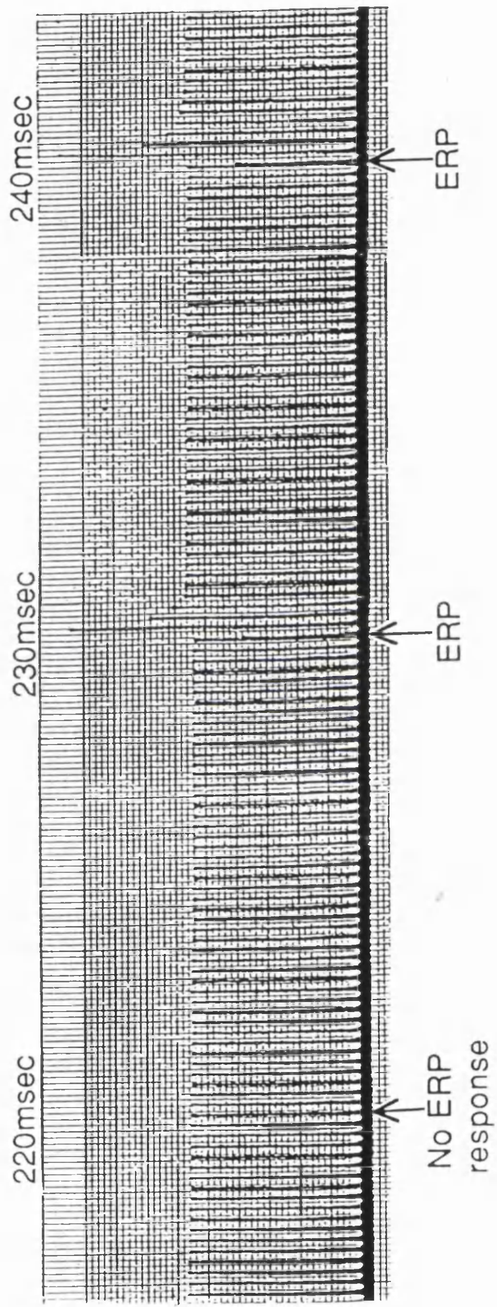

a)
+30mV
0
-90
Na+ in
Ca2+ in
K+ out
0
1
2
3
4
100msec

Figure 6.2 Effective refractory period (ERP) measurements in the paced guinea-pig papillary muscle preparation

This representative trace shows the stable contractile response of this preparation during continuous stimulation at a frequency of 1Hz. The duration of the ERP was determined by introducing a second additional electrical impulse at increasing intervals (220, 230, 240msec) following the preceding impulse. The initiation of an additional twitch response by this second stimulus indicated that the action potential had repolarised beyond the ERP (see Figure 6.1). In the example shown, no additional response was observed at 220msec but the ERP response did occur at 230msec. Note that when the additional twitch response occurred it was followed by a potentiated response.


240msec
230msec
220msec
ERP
ERP
No ERP response

6.2 METHODS

6.2.1 The guinea-pig perfused heart preparation

The paced perfused hearts were prepared as described in Section 2.3.1.

6.2.2 The guinea-pig papillary muscle preparation

The papillary muscles were prepared as described in Section 2.3.2. In addition to changes in contractile force, the effects on action potential duration were determined indirectly by measuring changes in the effective refractory period (ERP) in this preparation. All contractile force and effective refractory period measurements were expressed as a % of the measurement made at the end of the 60 minutes equilibration period unless stated otherwise.

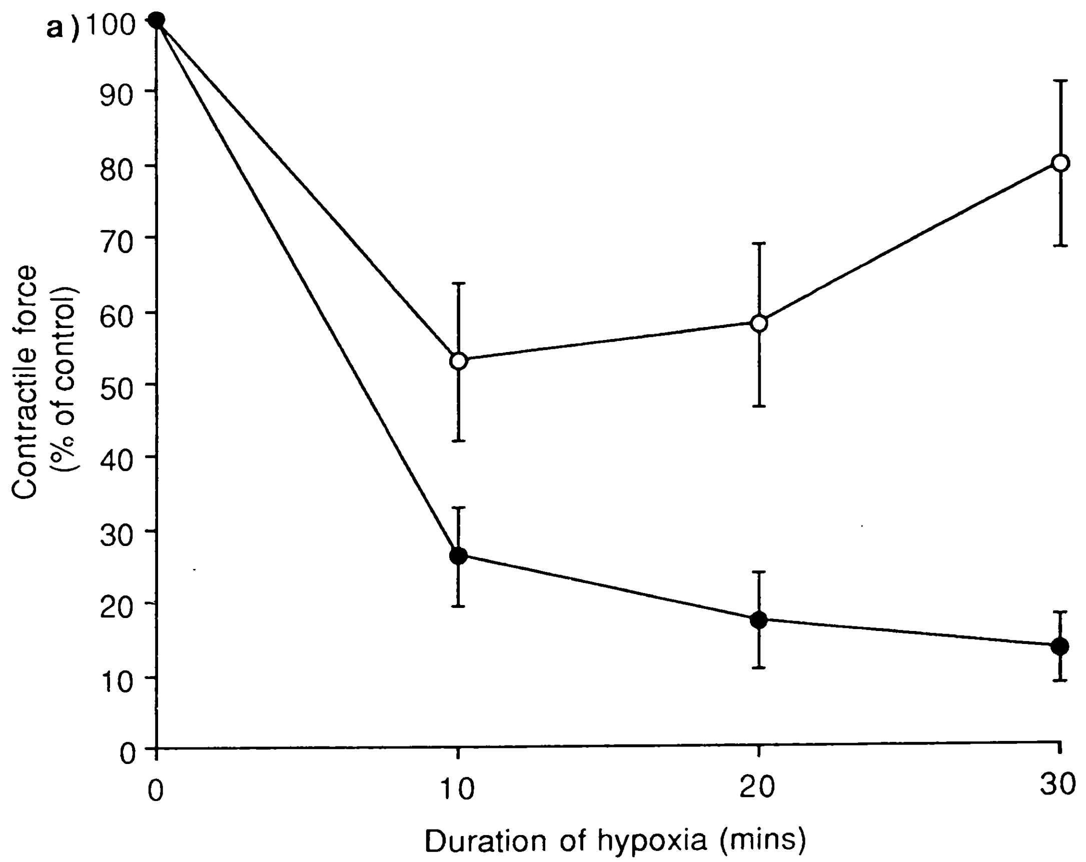
The ERP (Figure 6.1) has been defined as the period between action potentials at the end of which a second stimulus can induce a propagated mechanical response The ERP was measured using the following procedure.

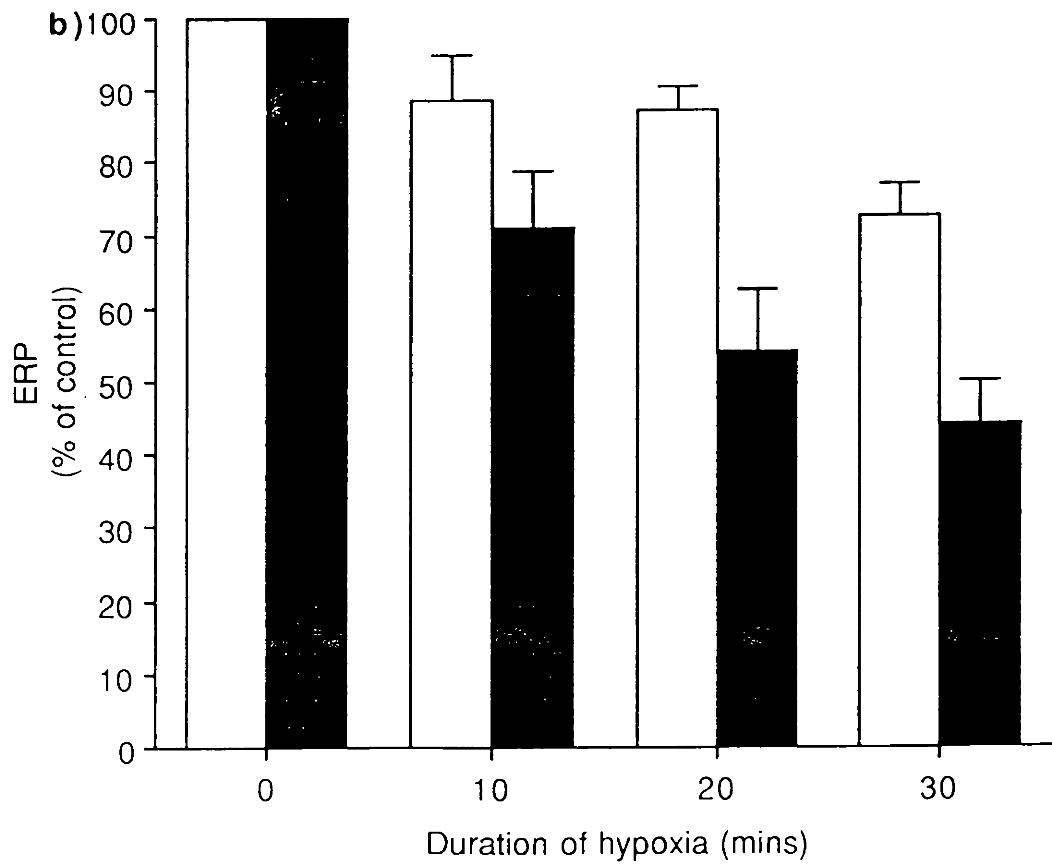
6.2.2.1 Measurement of the effective refractory period

ERP measurements were made by applying a second additional electrical impulse of identical strength to that applied during the normal 1Hz stimulation cycle. The timing of this extra impulse could be varied in 1msec steps and was electronically triggered by the preceding impulse. In this way, the time period between the normal 1Hz impulse and the extra stimulus could be increased until a second additional contraction was induced. This time period represented the ERP as previously defined and was usually between 170 and 250msec duration. A representative trace is shown in Figure 6.2.

Figure 6.3 The comparative effects of repeated hypoxia in the paced guinea-pig papillary muscle preparation

The effects of a single hypoxic insult (30 mins, open circles/bars n=5) followed by a second hypoxic insult (30 mins, closed circles/bars n=5) after a 60 minute oxygenated recovery period are shown. Changes to contractile force and ERP are presented in Figure a) and Figure b), respectively. The values shown are mean ± S.E. and are expressed as % of the measurements made immediately before each hypoxic insult.


a)
100
90
80
70
60
50
40
30
20
10
0
Contractile force
(% of control)
0
10
20
30
Duration of hypoxia (mins)


b)
100
90
80
70
60
50
40
30
20
10
0
ERP
(% of control)
0
10
20
30
Duration of hypoxia (mins)

6.2.2.2 Experimental conditions in hypoxic studies using the paced guinea-pig papillary muscle preparation

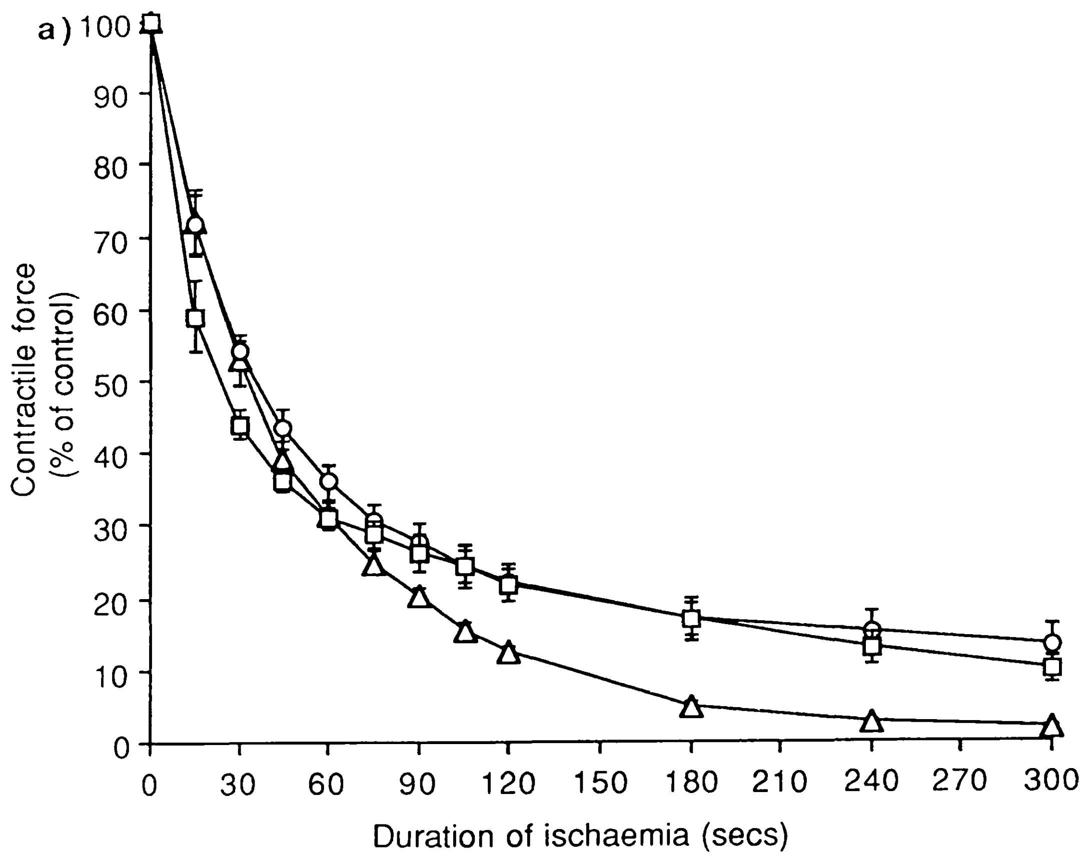
Hypoxia was induced by superfusion of the papillary muscles with PSS in which equimolar mannitol was substituted for glucose and the oxygen tension was reduced by gassing with 95%N_2:5%CO_2. Initial experiments showed that the response to a single 30mins hypoxic period was not marked and may have been insufficient to demonstrate any protective effects of drug intervention. However, if the tissues were allowed a 60 minutes recovery period of oxygenated glucose perfusion, the effects of a second hypoxic insult were more pronounced (Figure 6.3).

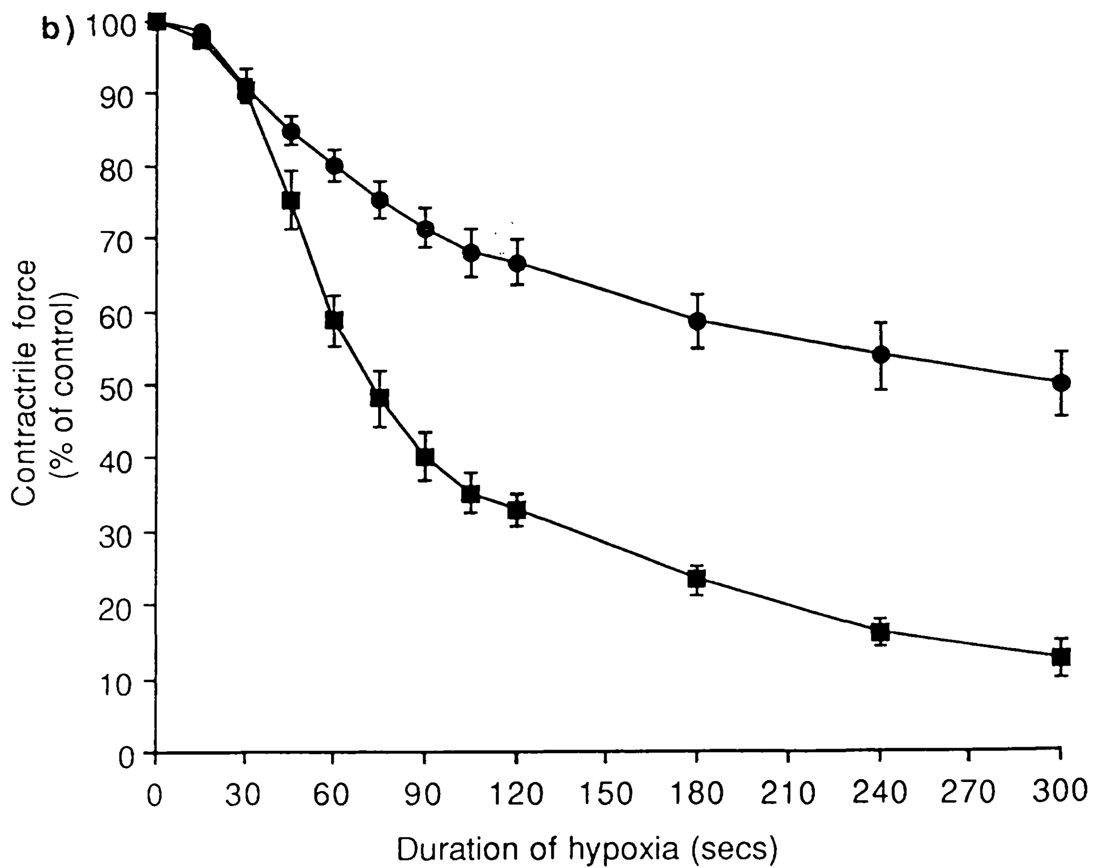
At the end of the 60 mins equilibration period, the ERP was measured. Hypoxia was introduced and the ERP was measured at 10 mins intervals for 30 mins. The tissues were re-oxygenated and allowed to recover for 60 mins. The ERP was measured at 40 and 60 mins during this recovery period at the end of which a second 30 mins hypoxic period was started. As before, the ERP was measured at 10 mins intervals throughout the hypoxic period. The contractile measurements were made prior to each ERP determination. When appropriate, the tissues were pre-treated with glibenclamide (20 mins) during the recovery period, as shown below. The drug was present throughout the second hypoxic period. In the absence of drug, normal perfusion was continued during these periods.

Equilibration	Hypoxia 1	Recovery	Drug	Hypoxia 2
60 mins	30 mins	40 mins	20 mins	30 mins

Figure 6.4 Early contractile failure (ECF) in response to ischaemia and hypoxia in the paced perfused guinea-pig heart

Measurements of the decrease in contractile force during 5 minutes of no-flow ischaemia (open triangles, n=7), low-flow ischaemia (open circles, n=6) and glucose-free low-flow ischaemia (open squares, n=6) are shown in a). The corresponding measurements during hypoxia (filled circles, n=4) and glucose-free hypoxia (filled squares, n=5) are shown in b). Values are mean ± S.E. and are expressed as % of the measurements made prior to the introduction of ischaemia or hypoxia.


a)
Contractile force
(% of control)
0
10
20
30
40
50
60
70
80
90
100
0
30
60
90
120
150
180
210
240
270
300
Duration of ischaemia (secs)


b)
Contractrile force
(% of control)
0
10
20
30
40
50
60
70
80
90
100
0
30
60
90
120
150
180
210
240
270
300
Duration of hypoxia (secs)

6.3 RESULTS AND DISCUSSION

6.3.1 The ECF response during ischaemia and hypoxia in the paced perfused guinea-pig heart

Previous experiments in the isolated perfused guinea-pig heart (Chapters 3 and 4) have shown that the initial response to ischaemia was a rapid decline of contractile force within 5 minutes of either low-flow or no-flow ischaemia (Figure 6.4a). Since one factor in ECF may be the rapid emptying of the ventricular cavity leading to a loss of contractile force through decreased intraventricular pressure, the effects of hypoxia on ECF were also examined. Under hypoxic conditions, ECF was not so pronounced and although still relatively rapid in onset, the degree and rate of decline clearly differed from that of ischaemia (Figure 6.4b).

Under low-flow ischaemic conditions, contractile force decreased to 35.9 ± 2.5% of control within 60 seconds and to 13.4 ± 2.7% of control after 5 minutes. Under no-flow ischaemic conditions, contractile force decreased to 31.2 ± 1.8% and 1.7± 0.4% of control at these time-points, respectively (Figure 6.4a). Therefore, no-flow ischaemia was associated with a greater decrease of contractile force during the ECF period (0 - 5mins). During hypoxia (Figure 6.4b), contractile force was 80.0 ± 2.2% of control after 60 seconds and 49.6 ± 4.5% after 5 minutes.

For comparison t_{50} (time to 50% decrease of contractile force) values were calculated. The respective values for no-flow, low-flow and glucose-free low-flow ischaemia were 32.6 ± 3.4, 37.7 ± 2.7 and 22.8 ± 3.2 seconds and for hypoxia and glucose-free hypoxia were 156.0 ± 33.8 and 72.8 ± 4.9 seconds, respectively .

No significant differences were observed in the ECF responses during low-flow ischaemia with and without glucose (Figure 6.4a). However, the response to hypoxia was shifted towards that of low-flow ischaemia by the withdrawal of glucose (Figure 6.4b). After 60 seconds of glucose-free hypoxia, contractile force

Figure 6.5 The effect of glibenclamide on cardiac function during normoxic perfusion in the paced perfused guinea-pig heart

This representative trace shows the effect of 3mcM glibenclamide on a) coronary perfusion pressure and b) contractile force in the normoxic perfused heart. The control perfusion pressure was 40mmHg and contractile force was approximately 0.7g prior to the addition of glibenclamide.

a)

b)

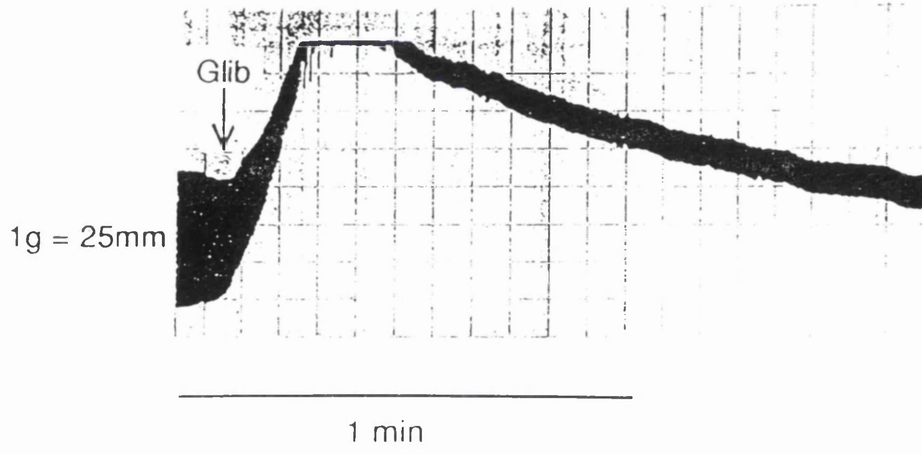
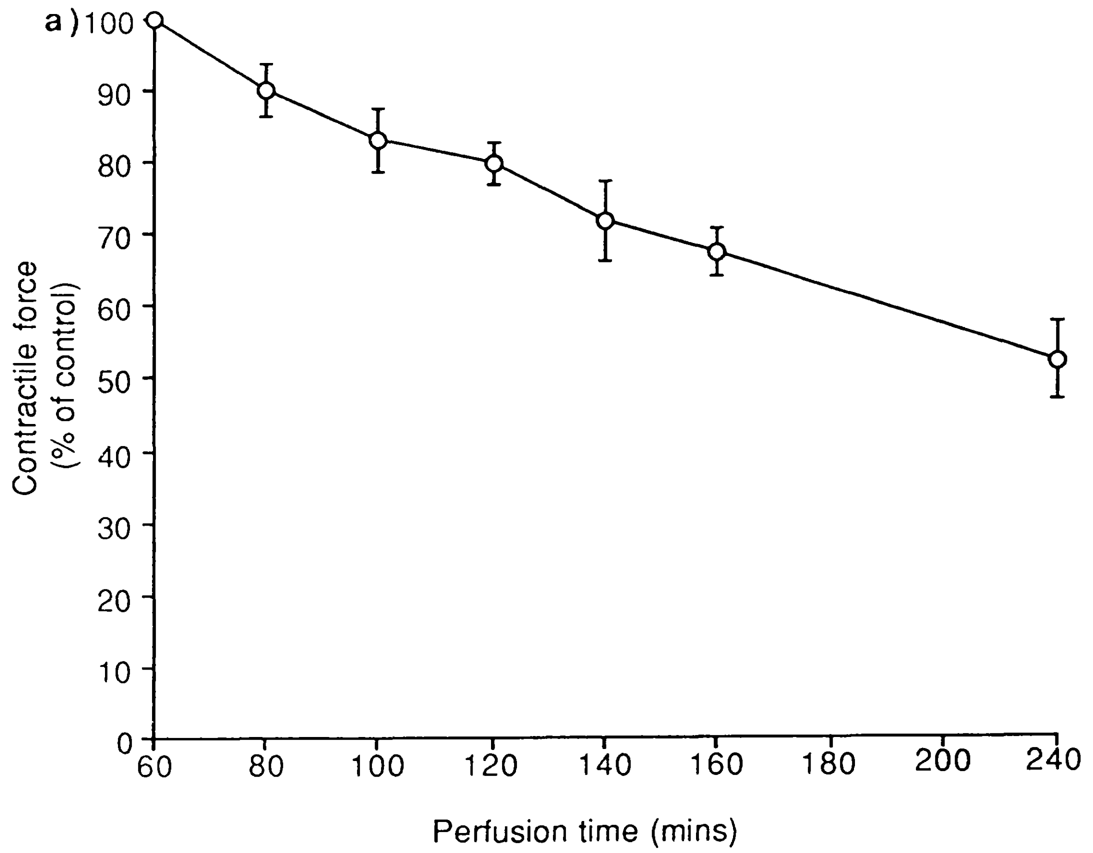
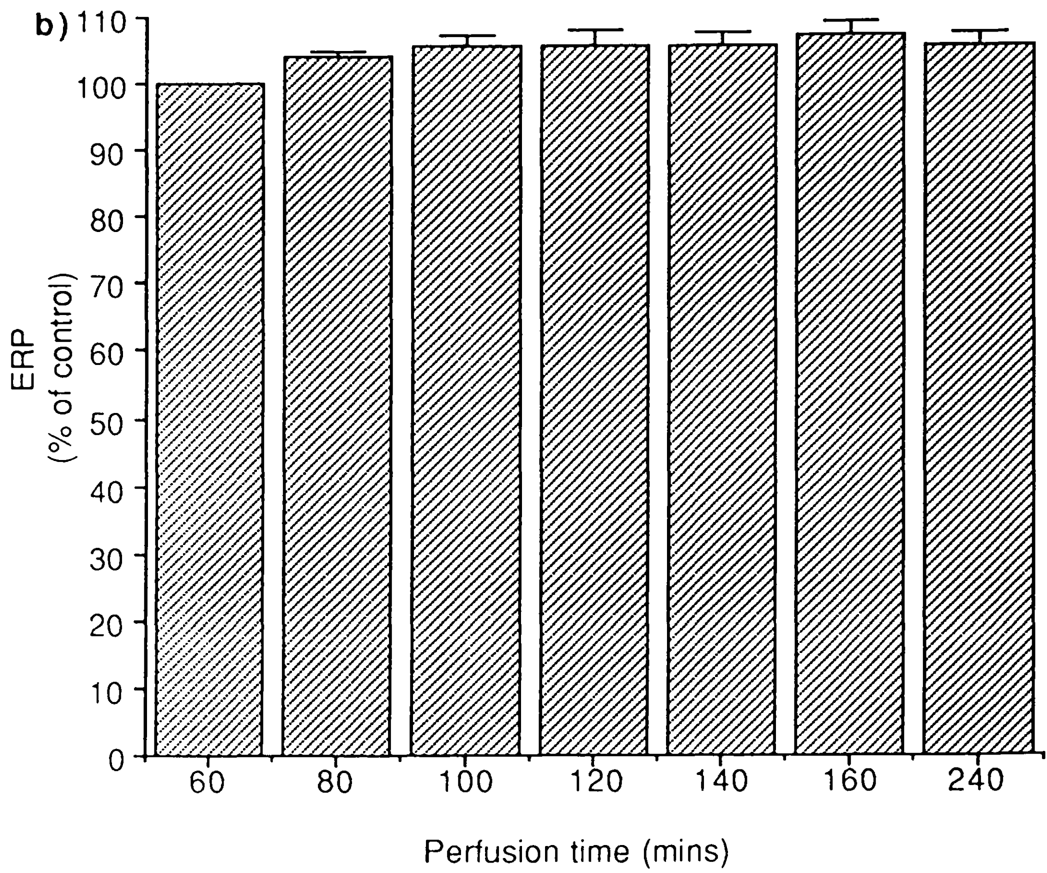


Figure 6.6 Time-dependent changes of contractile force and ERP in the paced guinea-pig papillary muscle preparation superfused under normoxic conditions

The effects of perfusion time on contractile force (n=5) and ERP (n=5) are shown in Figures a) and b), respectively. Contractile force and ERP were measured at the end of the 60 minute equilibration period (60 mins). All subsequent values are expressed as % of this measurement. All values shown are mean ± S.E.


a)
Contractile force
(% of control)
100
90
80
70
60
50
40
30
20
10
0
60
80
100
120
140
160
180
200
240
Perfusion time (mins)


b)
ERP
(% of control)
110
100
90
80
70
60
50
40
30
20
10
0
60
80
100
120
140
160
240
Perfusion time (mins)

had decreased to 58.6 ± 3.5% of control and was 12.4 ± 2.7% of control after 5 minutes. While it was not possible to eliminate the effects of loss of ventricular pressure as a contributing factor in the ischaemic experiments, glucose utilisation was clearly of major importance in the ECF response during hypoxia.

6.3.2 The effect of glibenclamide in the paced perfused guinea-pig heart

Perfusion with 10μM glibenclamide, a concentration which has been shown to reduce K^+ loss during ischaemia in the perfused rat heart (Kantor *et al.*, 1990), caused a rise in perfusion pressure in the guinea-pig model. This was also the case for 3μM glibenclamide (Figure 6.5a). Since this effect of glibenclamide was also associated with impaired contractile function (Figure 6.5b), it was decided to do all further investigations on K_{ATP} activity and contractile force using isolated papillary muscles subjected to hypoxia.

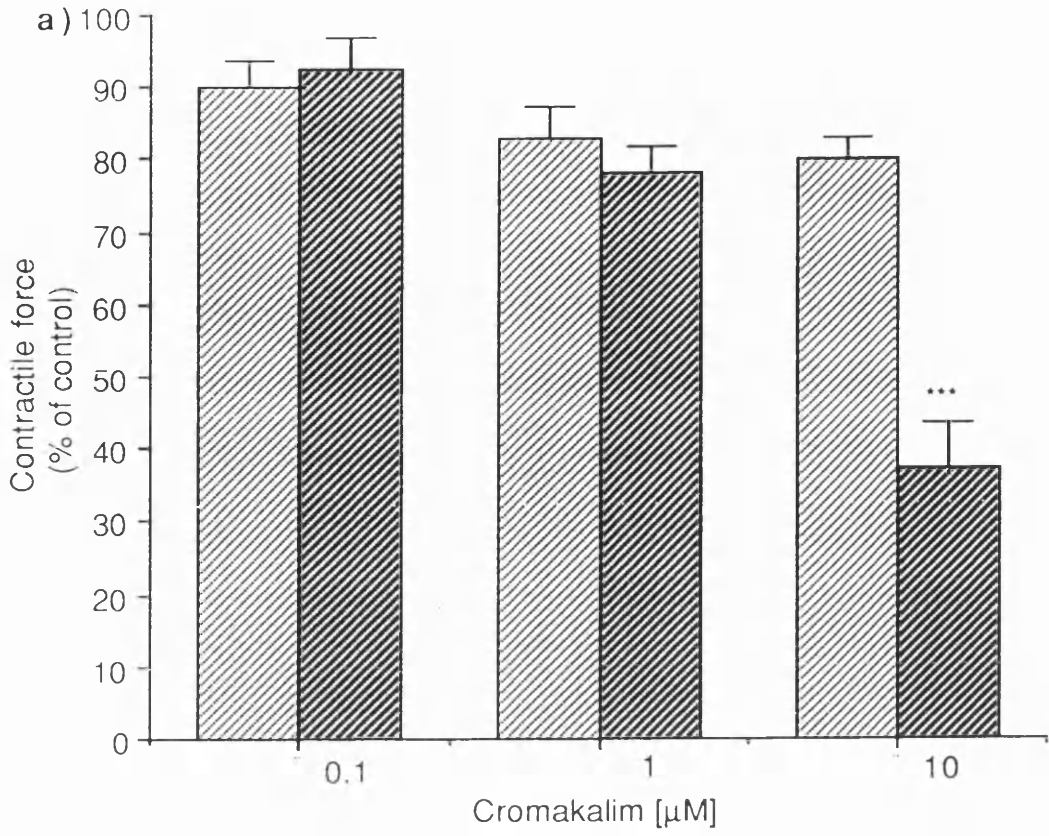
6.3.3 The effects of cromakalim in the paced guinea-pig papillary muscle preparation

To demonstrate K_{ATP}-channel activation in this preparation, the effects of the K_{ATP}-channel opener, cromakalim, were examined. Time-matched control values obtained from tissues perfused over the same experimental duration in the absence of drug showed that this preparation exhibits a time-dependent "run-down" of contractile force during superfusion (Figure 6.6). The ERP, however, remained stable during the experiment and was 200 - 250msec which was within the normal range for cardiac muscle (Katz, 1992).

The effects of 0.1, 1 and 10μM cromakalim on contractile force and the ERP are compared in Figure 6.7. Superfusion for 20 minutes with 0.1 and 1mcM cromakalim had no significant effects on contractile force or the ERP. A higher concentration of cromakalim (10μM) reduced contractile force to 37.1 ± 6.3% and

Figure 6.7 The effects of cromakalim on contractile force and ERP in the paced guinea-pig papillary muscle preparation perfused under normoxic conditions

The effects of 0.01, 1 and 10μM cromakalim (dark hatch bars), on contractile force (Figure a, n=5) and ERP (Figure b, n=5) were compared with time-matched responses obtained from normoxic-perfused papillary muscles (light hatch bars, n=5) in the absence of drug. Each concentration was perfused sequentially for 20 minutes. All measurements were made at the end of each 20 minute period and the values shown are mean ± S.E. expressed as % of the control measurement made at 60 minutes as described in the legend for Figure 6.6. Statistical analysis was by analysis of variance and application of a two-sided t-test (*** $p < 0.001$)


a)
Contractile force
(% of control)
100
90
80
70
60
50
40
30
20
10
0
***
0.1
1
10
Cromakalim [μM]

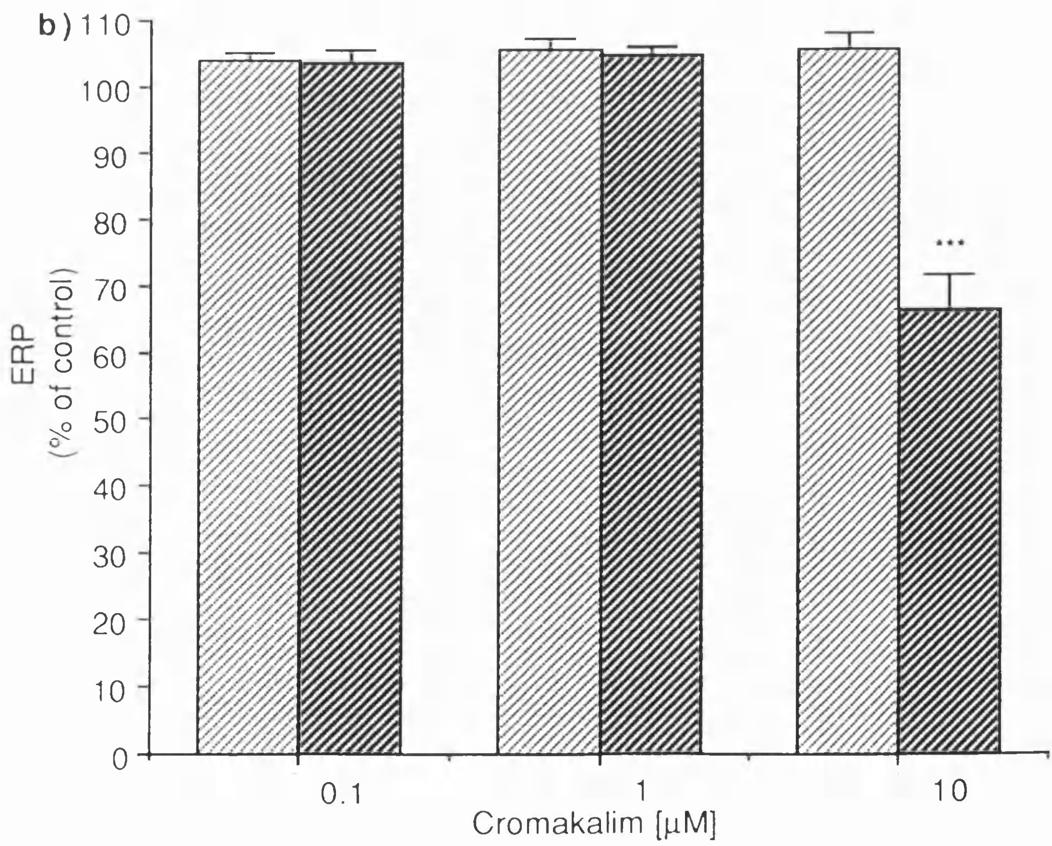
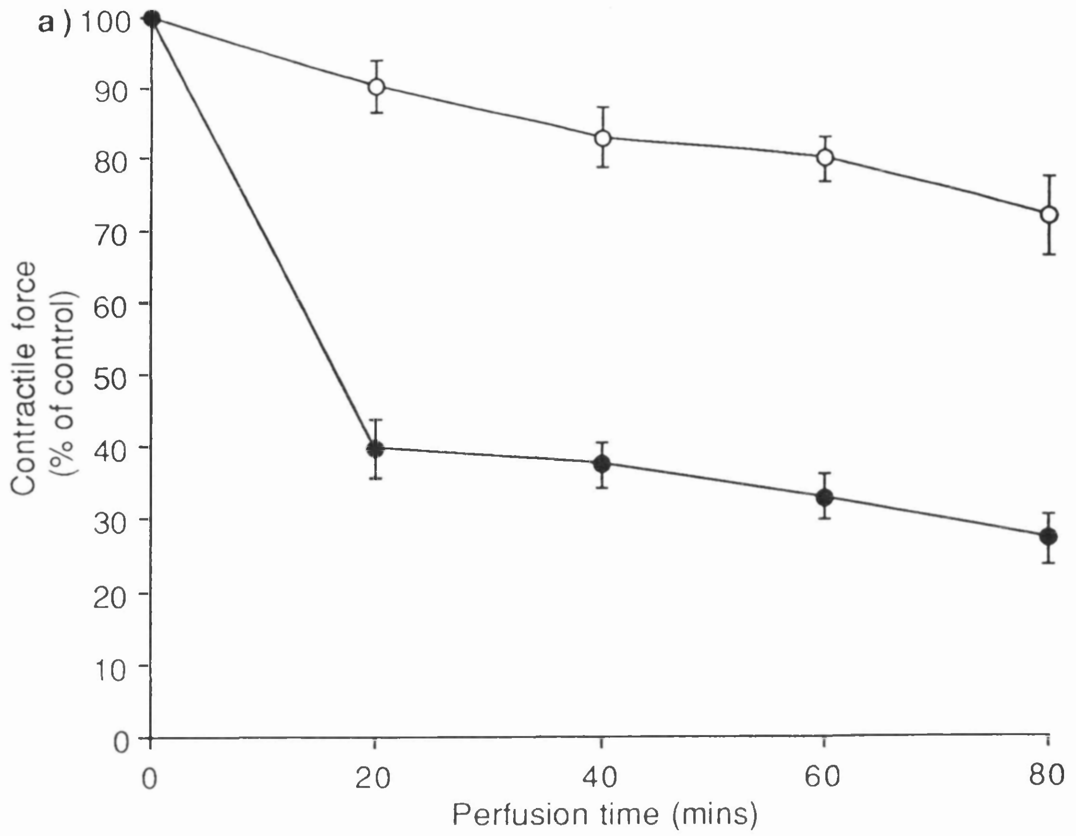
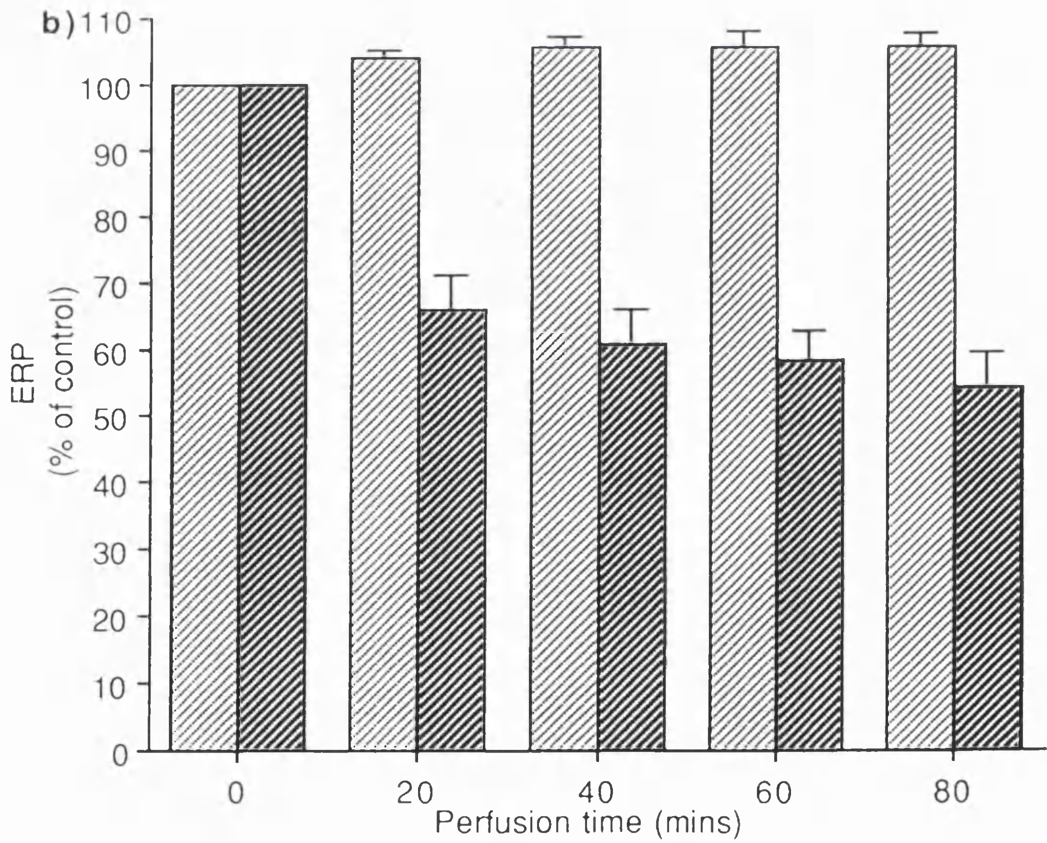

b)
ERP
(% of control)
110
100
90
80
70
60
50
40
30
20
10
0
***
0.1
1
10
Cromakalim [μM]

Figure 6.8 Time-dependent effects of cromakalim on contractile force and ERP in the paced guinea-pig papillary muscle preparation superfused under normoxic conditions

This figure shows the effects of prolonged superfusion with a single concentration of 10μM cromakalim (closed circles/dark hatch bars, n=4) on a) contractile force and b) ERP. These measurements were compared with time-matched responses (open circles/light hatch bars, n=5) obtained from papillary muscles superfused under normoxic conditions in the absence of drug. All values are mean ± S.E. expressed as described in the legend for Figure 6.6.


a)
Contractile force
(% of control)
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
Perfusion time (mins)


b)
ERP
(% of control)
110
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
Perfusion time (mins)

the ERP to 66.1 ± 5.4% of control . The corresponding time-matched values for contractile force and ERP at these time-points were 79.6 ± 3.0% and 105.5 ± 2.4%, respectively. The effects of cromakalim were not increased by prolonged superfusion (Figure 6.8). These results suggested that the shortening of the ERP was associated with a negative inotropic effect. If both events were the result of K_{ATP}-channel activation, then these effects should be reversed by glibenclamide.

6.3.4 Inhibition of the effects of cromakalim by glibenclamide in the paced guinea-pig papillary muscle preparation.

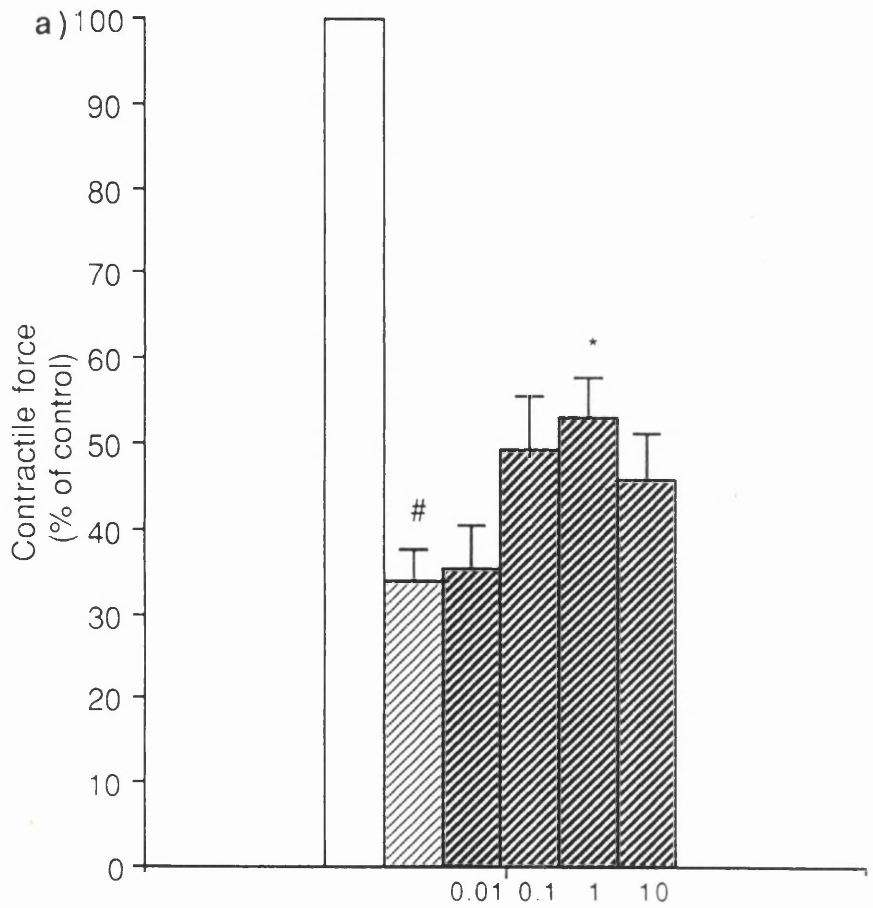
Superfusion for 30 minutes with a single concentration of 10µM cromakalim resulted in a decrease of contractile force to 34.1 ± 3.7% of control and the ERP was shortened to 51.3 ± 3.7% of control (Figure 6.9). Cromakalim was present throughout the remainder of the experiment and each glibenclamide concentration was superfused fro 20 minutes. While the effect on the ERP was partially reversed to 79.0 ± 2.9% of control by 0.1µM glibenclamide and almost completely reversed to 94.3 ± 1.9% and 97.7 ± 1.4% of control by 1 and 10µM glibenclamide, respectively, the effects of cromakalim on contractile force were not fully reversed (Figure 6.9). This indicated that the effects on the ERP and contractile force could be dissociated.

The "run-down" effect already described (see section 6.3.3) may have contributed to the lack of reversabilty of contractile force since over the duration of this experiment a decrease to around 65% of control would be expected (extrapolated from the data shown in Figure 6.6).

A non-specific action of cromakalim and glibenclamide on contractile force was also considered. However, in a further set of experiments, it was found that superfusion with 10µM glibenclamide alone for 20 minutes did not effect contractile force or the ERP. Pre-treatment with glibenclamide was effective in preventing the subsequent effects of cromakalim (Figure 6.10). The decrease in contractile force

Figure 6.9 The effect of glibenclamide on the response to cromakalim in the paced guinea-pig papillary muscle preparation superfused under normoxic conditions

This figure shows the effects of 0.01, 0.1, 1 and 10μM glibenclamide (dark hatch bars, n=5), superfused sequentially for 20 minutes at each concentration, on changes to a) contractile force and b) ERP following 30 minutes perfusion with 10μM cromakalim (light hatch bars, n=5). For reference, control levels are indicated by white bars. All values are mean ± S.E expressed as described in the legend for Figure 6.6. Statistical comparison of the control group and cromakalim (mm or msec values) was by unpaired t-test (# $p < 0.01$ compared to control value). The effects of glibenclamide on the response to cromakalim (% values) were compared using analysis of variance and application of Dunnett's t-test (* $p < 0.05$ compared to cromakalim, ** $p < 0.01$ compared to cromakalim).


a)
Contractile force
(% of control)
100
90
80
70
60
50
40
30
20
10
0
#
*
0.01
0.1
1
10

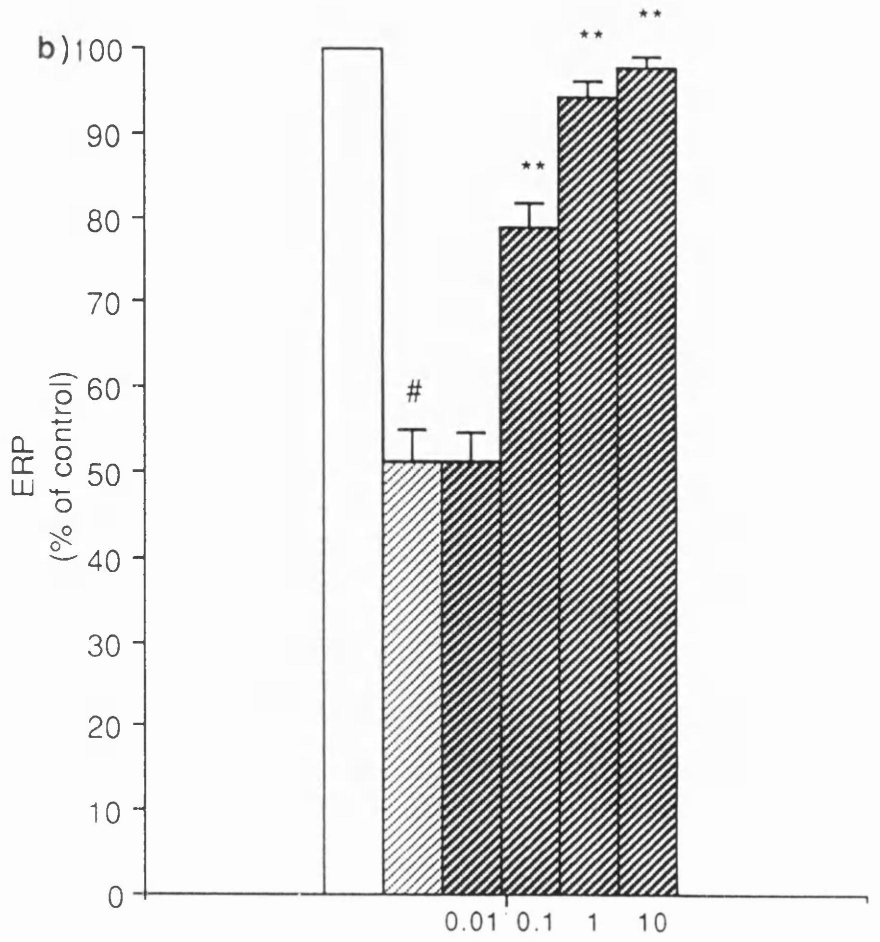
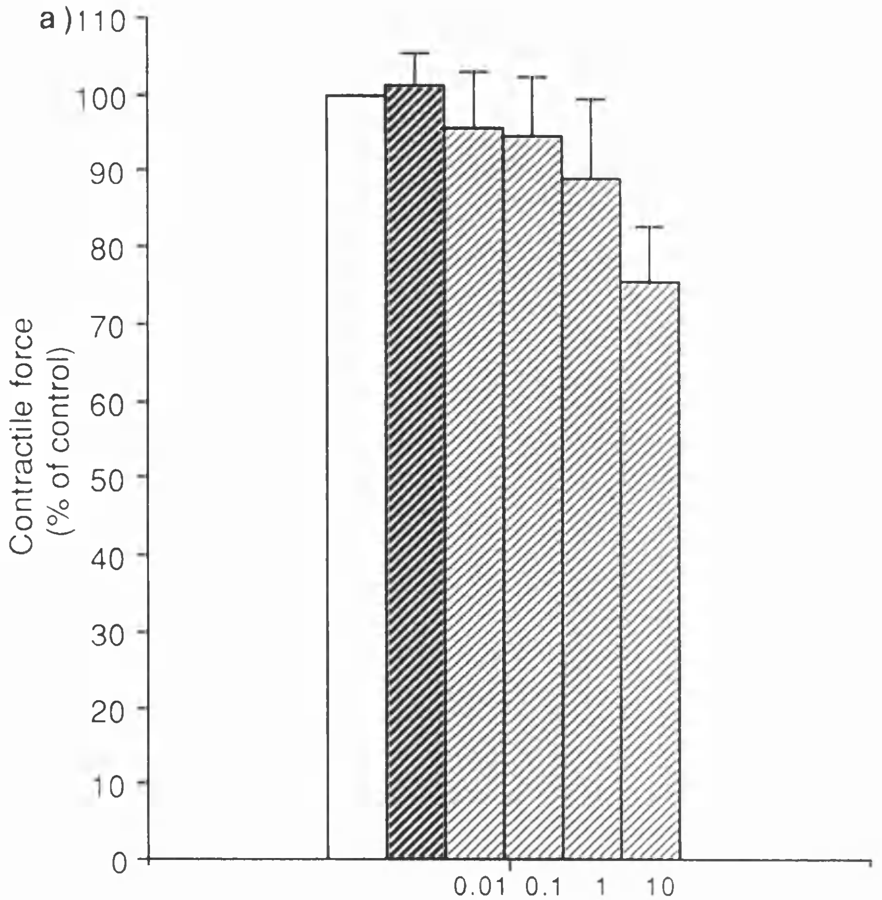
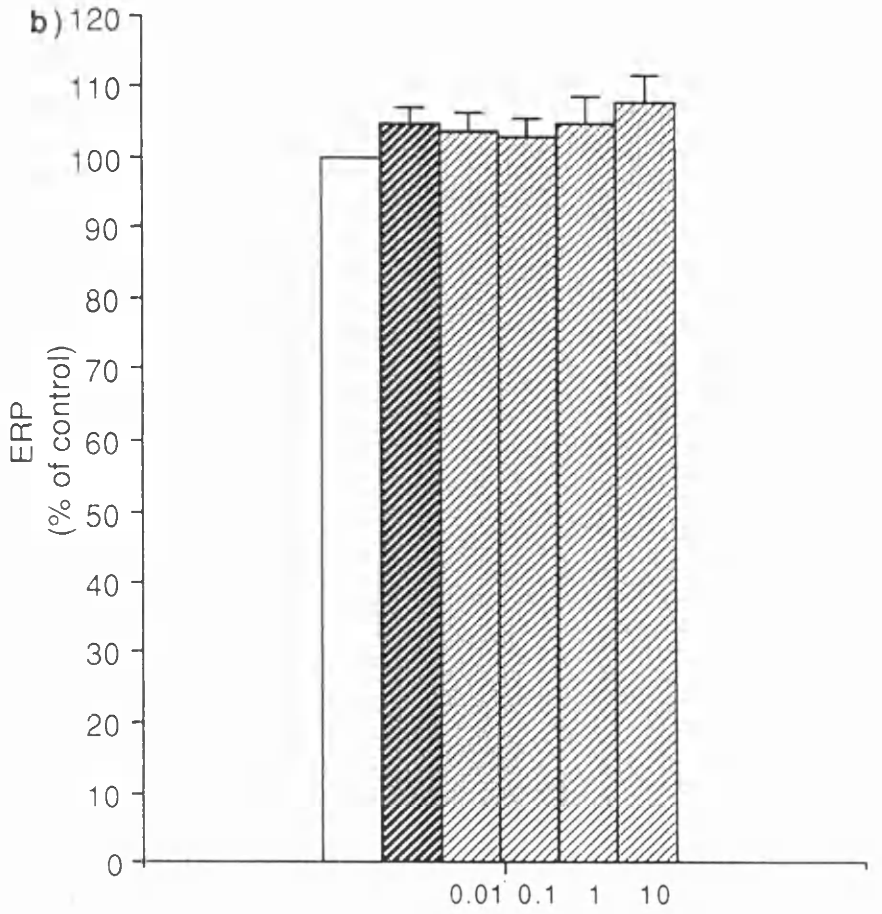

b)
ERP
(% of control)
100
90
80
70
60
50
40
30
20
10
0
#
**
**
**
0.01
0.1
1
10

Figure 6.10 The effects of cromakalim on contractility and ERP following pre-treatment with glibenclamide in the paced guinea-pig papillary muscle preparation superfused under normoxic conditions

This figure shows the effects of 0.01, 0.1, 1 and 10μM cromakalim (light hatch bars, n=5), perfused sequentially for 20 minutes at each concentration, on changes to a) contractile force and b) ERP following 20 minutes perfusion with 10μM glibenclamide (dark hatch bars, n=5). For reference, control levels are indicated by white bars. All values are mean ± S.E expressed as described in the legend for Figure 6.6. Statistical comparison between the control group and glibenclamide (mm or msec values) was by unpaired t-test. The effects of glibenclamide on the response to cromakalim were compared using analysis of variance and application of Dunnett's t-test. No significant differences were detected between the control and glibenclamide or between the glibenclamide and cromakalim groups.


a)
Contractile force
(% of control)
110
100
90
80
70
60
50
40
30
20
10
0
0.01
0.1
1
10


b)
ERP
(% of control)
120
110
100
90
80
70
60
50
40
30
20
10
0
0.01
0.1
1
10

to 67.2 ± 3.3% of control during this experiment was similar to that expected of the "run-down" effect. The " run-down" of contractile force in this preparation does not appear to be associated with changes in ERP.

6.3.5 The effects of glibenclamide on the response to hypoxia in the paced guinea-pig papillary muscle preparation.

In the studies on the effects of hypoxia, all measurements were expressed as a % of the 40 mins recovery value (see section 6.2.2.2.)

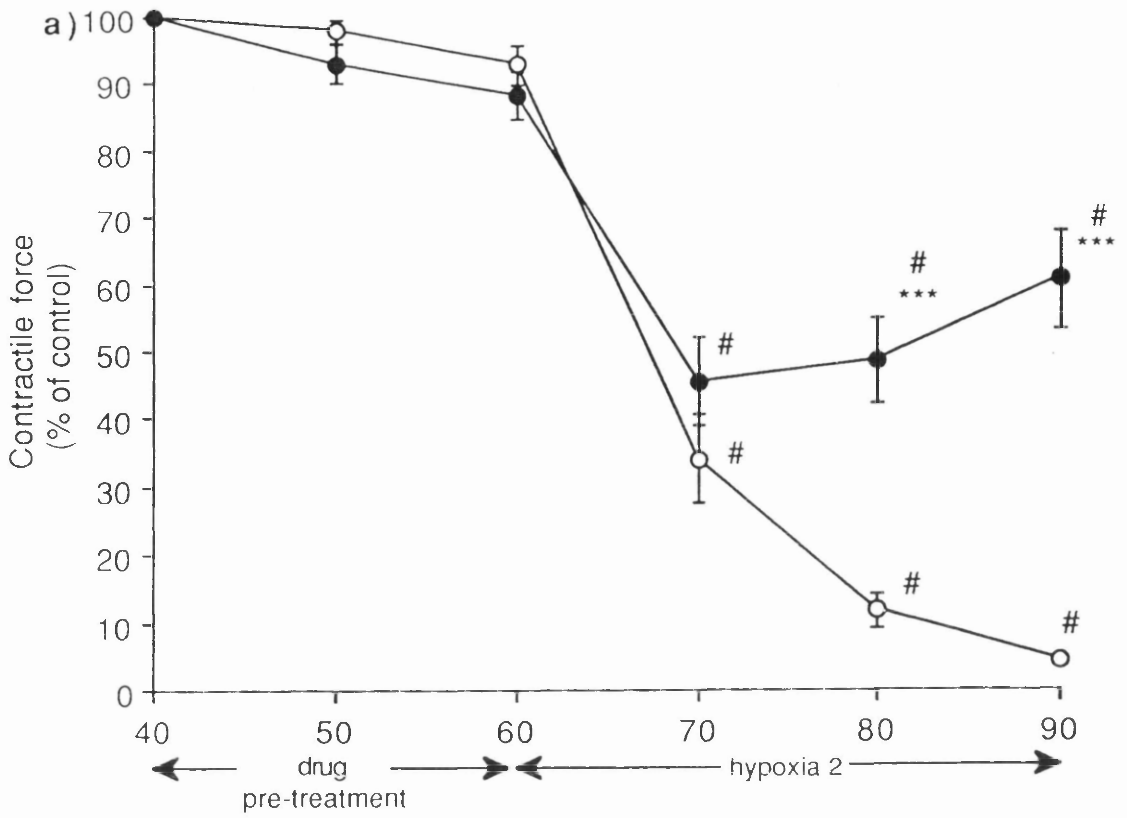
In this series of experiments, hypoxia caused a time-dependent decrease in contractile force and shortened the ERP (Figure 6.11). These effects were most rapid in the initial 10 minutes of hypoxia during which contractile force decreased from 92.7 ± 2.9% to 33.9 ± 6.5% of control and ERP shortened from 100.6 ± 0.6% to 57.0 ± 3.4% of control. At the end of 30 minutes hypoxia, contractile force was 4.5 ± 0.6% and the ERP was 34.6 ± 4.5% of control.

The effects of glibenclamide pre-treatment were compared to time-matched measurements obtained from tissues superfused with normal PSS. Pre-treatment (20 mins) with glibenclamide had no significant effect on contractile force or ERP (Figure 6.11a and b). Glibenclamide attenuated the effect of hypoxia on the ERP which shortened from 102.8 ± 0.9% to 75.5 ± 2.9% of control over 30 minutes of hypoxia. During the first 10 mins of hypoxia, contractile force decreased from 88.2 ± 3.4% to 45.3 ± 6.5% of control. This effect was not different from that of hypoxia alone. However, after this timepoint, contractile force began to increase and was 60.6 ± 7.3% of control at the end of the hypoxic period.

In order to determine whether this improvement in contractile activity was reflected in changes to ATP depletion, the ATP content of the tissues was measured. The papillary muscles were frozen in liquid nitrogen at the end of the hypoxic period and the ATP content was measured as described in Section 2.3.4. The normoxic ATP content was measured in papillary muscles perfused under oxygenated conditions over the same duration.

Figure 6.11 The effects of glibenclamide on the response to hypoxia in the paced guinea-pig papillary muscle preparation

When used, glibenclamide (10μM) was superfused for 20 minutes over the period between 40 and 60 minutes of oxygenated recovery following the first hypoxic insult (see section 6.2.2.2) and also throughout the second hypoxic period. The effects of hypoxia (open circles/light hatch bars, n=7-21) and hypoxia + glibenclamide (closed circles/dark hatch bars n=20-22) on contractile force and ERP during the second hypoxic insult are shown in a) and b), respectively. All values are mean ± S.E. expressed as % of the measurements made prior to the addition of glibenclamide. Statistical analysis of the effect of hypoxia alone was by analysis of variance and application of Dunnett's t-test (# $p< 0.01$ compared to control value). The effect of glibenclamide on hypoxia was evaluated using a non-parametric Mann-Whitney test (*** $p< 0.001$ compared to the corresponding hypoxic value).


a)
Contractile force
(% of control)
100
90
80
70
60
50
40
30
20
10
0
#
***
#
***
#
#
#
#
40
50
60
70
80
90
drug
pre-treatment
hypoxia 2

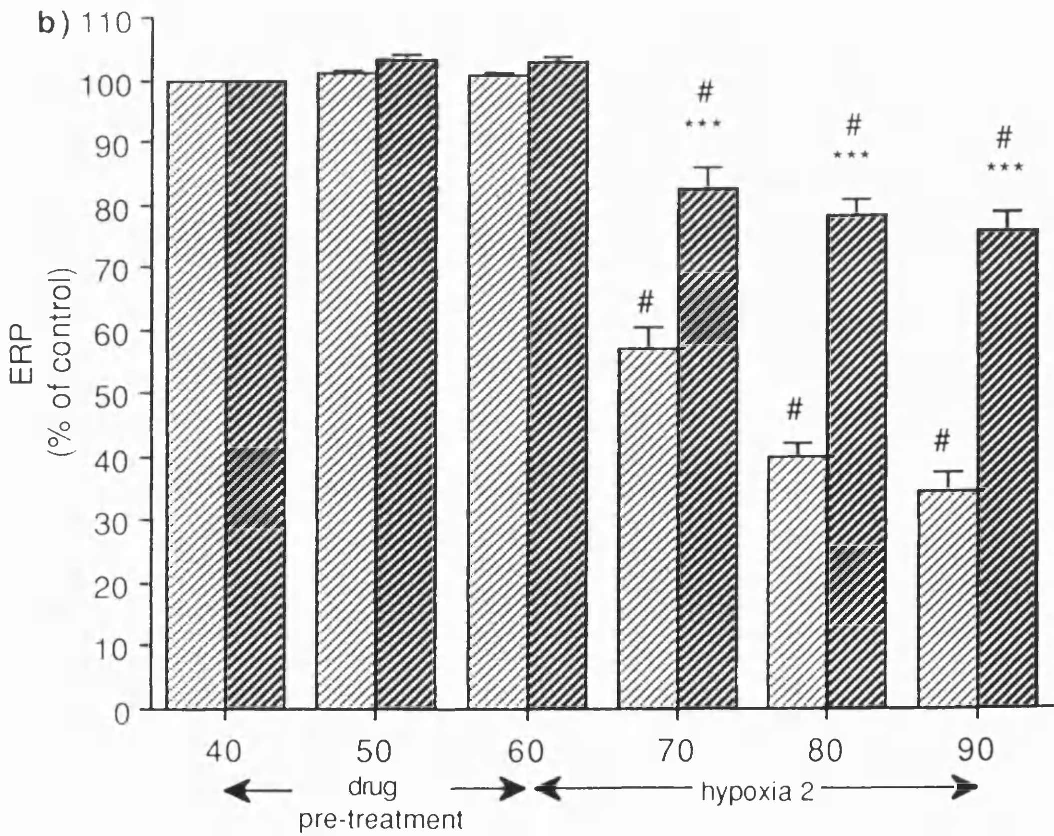
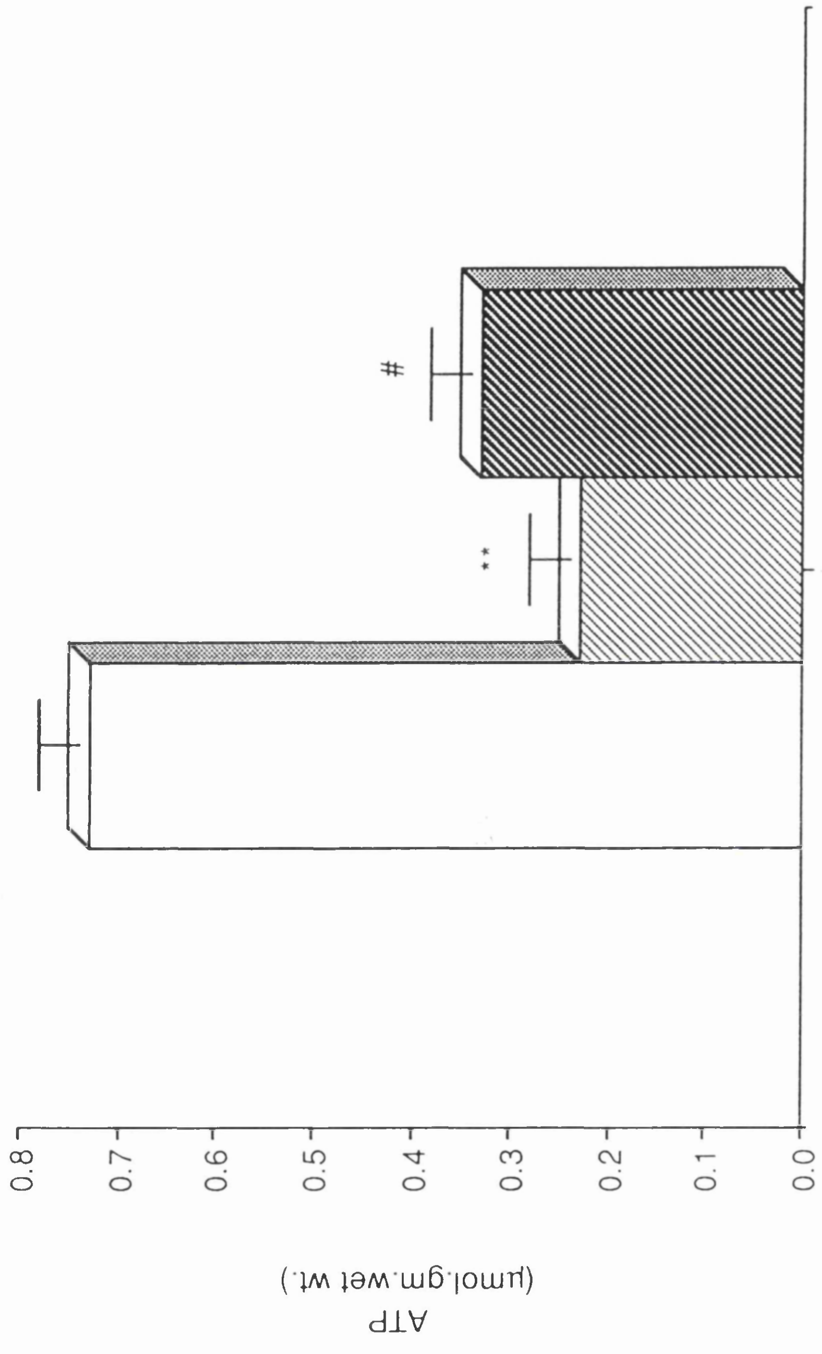

b)
ERP
(% of control)
110
100
90
80
70
60
50
40
30
20
10
0
#
***
#
***
#
***
#
#
#
40
50
60
70
80
90
drug
pre-treatment
hypoxia 2

Figure 6.12 The effects of glibenclamide on ATP depletion during hypoxia in the paced guinea-pig papillary muscle preparation

ATP levels were measured in papillary muscles subjected to 30 minutes of hypoxia with and without 10μM glibenclamide treatment as shown in Figure 6.11. A group of time-matched tissues superfused under normoxic conditions in the absence of glibenclamide were included for comparison. The values shown are mean ± S.E. and represent the levels determined in pooled groups of 3-6 tissues. Statistical comparison between time-matched normoxia (open bar, n=4 groups; 5 muscles/group), hypoxia (light hatch bar, n=5; 3-5 muscles/group) and hypoxia + glibenclamide (dark hatch bar, n=4; 5-6 muscles/group) was by analysis of variance and application of Dunnett's t-test. (** $p< 0.01$ compared to normoxia; # not significantly different from hypoxia).



ATP
(µmol.gm.wet wt.)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
#

Normoxic ATP concentrations were 0.7 ± 0.04μmol/gm wet wt. After 30 mins of hypoxia alone, ATP was significantly reduced to 0.2 ± 0.04μmol/gm wet wt. In the presence of glibenclamide, the reduction of ATP to 0.3 ± 0.06μmol/gm wet wt was not significantly different to that of hypoxia alone (Figure 6.12).

Glibenclamide did not affect the ECF response to hypoxia but was associated with an improved contractile response later in the hypoxic period. ATP depletion was similar in both hypoxic groups.

6.3.6 The effect of glibenclamide on the response to prolonged hypoxia in the paced guinea-pig papillary muscle preparation

The effects of glibenclamide on contractile force and ERP shortening were investigated over a longer duration of hypoxia (90 mins). These results are shown in Figure 6.13.

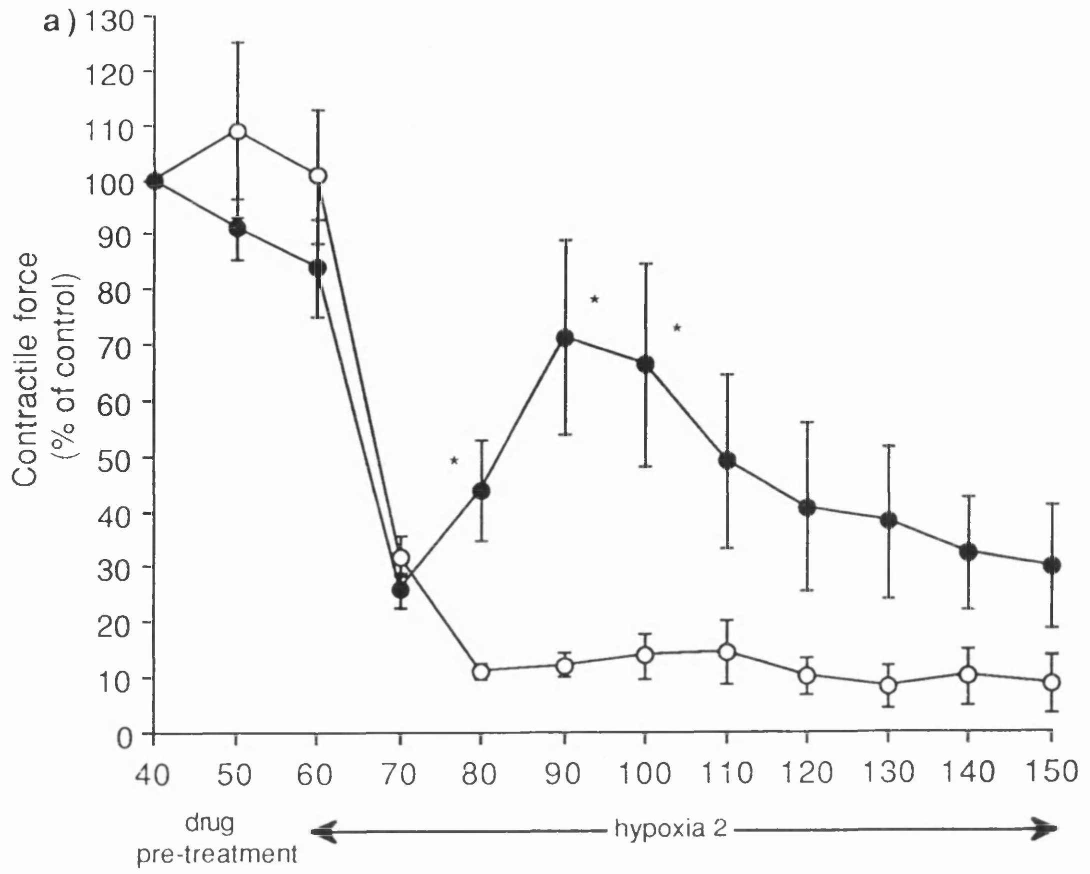
As observed in the previous experiment, 20 mins pre-treatment with 10μM glibenclamide had no significant effects on contractile force or the ERP. At the end of the pre-treatment period, contractile force and the ERP were 83.8 ± 9.0% and 105.8 ± 3.3% of control, respectively, compared to 100.5 ± 12.1% and 103.3 ± 2.1% of control in the absence of drug.

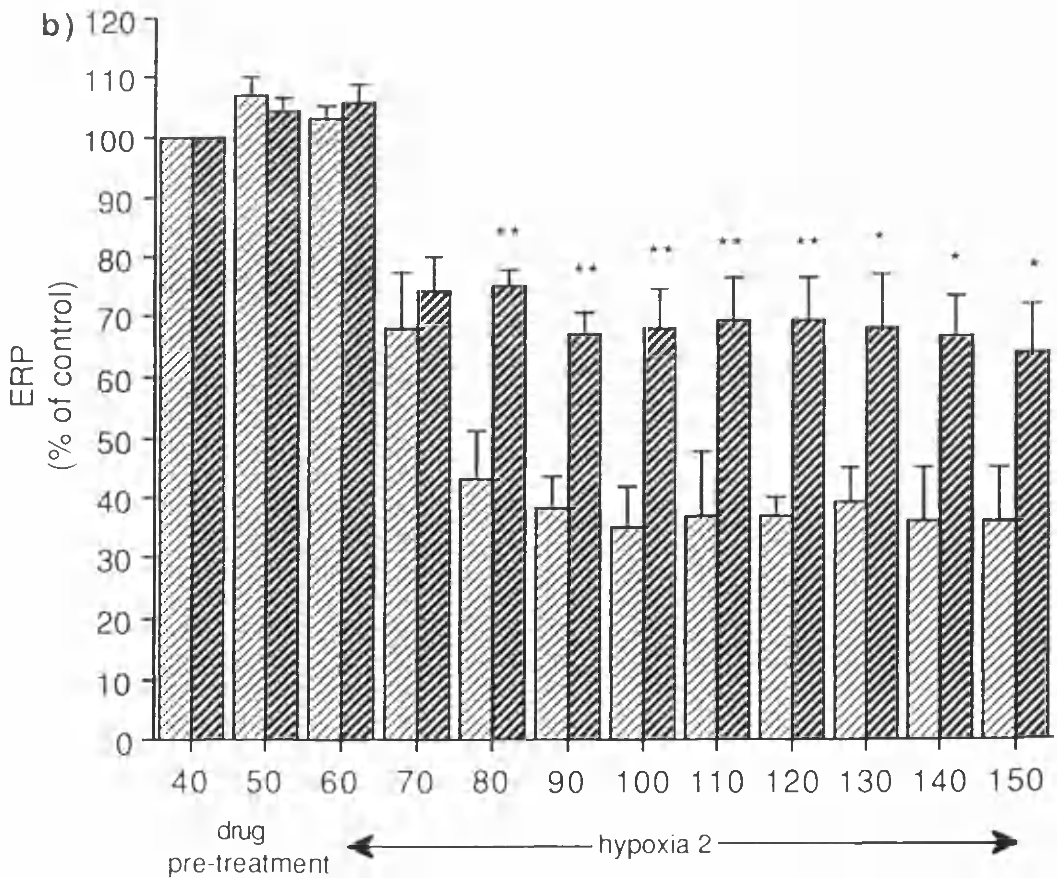
In the presence of glibenclamide, the ERP shortened from 105.8 ± 3.3% to 74.2 ± 5.6% of control during the initial 10 mins of hypoxia. This was not significantly different from the response to hypoxia alone. After this time, as in the previous experiment, the rate and degree of ERP shortening in the presence of glibenclamide was less than that in hypoxia alone. After 20 mins of hypoxia, the ERP had decreased to 75.0 ± 2.9% in the glibenclamide-treated group compared to 43.3 ± 7.8% of control in the absence of drug. At the end of the hypoxic period, the respective values for the ERP in each group were 63.8 ± 8.3% and 35.9 ± 9.2% of control.

The decrease in contractile force during the initial 10 mins of hypoxia was

Figure 6.13 The effects of glibenclamide on the response to prolonged hypoxia in the paced guinea-pig papillary muscle preparation

This experiment was as described in the legend for Figure 6.11 except that the hypoxic period was extended to 90 minutes. Hypoxia (open circles/light hatch bars, n=2-5); hypoxia + glibenclamide (closed circles/dark hatch bars, n=5). Hypoxia significantly reduced contractile force and shortened ERP as in the previous experiments shown in Figure 6.11. The effect of glibenclamide on hypoxia was evaluated using a non-parametric Mann-Whitney test (*$p < 0.05$, **$p<0.01$) compared to the corresponding hypoxic value). For clarity, only the statistical comparison between hypoxia and hypoxia + glibenclamide has been shown.


a)
Contractile force
(% of control)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
40
50
60
70
80
90
100
110
120
130
140
150
drug
pre-treatment
hypoxia 2


b)
ERP
(% of control)
0
10
20
30
40
50
60
70
80
90
100
110
120
40
50
60
70
80
90
100
110
120
130
140
150
drug
pre-treatment
hypoxia 2

similar in both groups. Hypoxia in the absence of drug caused a decrease in contractile force from 100.5 ± 12.1 to 31.9 ± 3.7% of control, while in the presence of glibenclamide contractile force decreased from 83.8 ± 8.9 to 25.7 ± 3.0% of control during this period. In the absence of glibenclamide, contractile force continued to decrease and was 8.7 ± 5.3% of control at the end of the hypoxic period. As in the previous experiment, glibenclamide prevented the progressive loss of contractile force and contractile force recovered to 71.2 ± 17.4% of control after 30 mins of hypoxia compared to 12.1 ± 2.2% of control during hypoxia alone. This protective effect of glibenclamide was transient and contractile force decreased again after 40 mins of hypoxia. Nevetherless, at the end of the hypoxic period, contractile force was 29.9 ± 11.0% of control in the presence of glibenclamide compared to 8.7 ± 5.3% of control after hypoxia alone.

6.4 DISCUSSION

Early contractile failure occurred in both low-flow and no-flow ischaemia and also in hypoxia in glucose-perfused hearts. The degree of ECF was greater during ischaemia than in hypoxia which probably reflects the potential of energy production from continued glycolysis in addition to the maintenance of intraventricular pressure during hypoxia. However, the loss of perfusion pressure can only partially account for ECF since these experiments have shown that removal of exogenous glucose during hypoxia resulted in a marked increase in the degree of ECF. This intervention was less effective in low-flow ischaemia where glycolytic flux is reduced.

Since H^+ and lactate accumulation would be expected to be less under glucose-free hypoxic conditions, these results suggest a major component of the ECF response involves glucose metabolism rather than the loss of intraventricular pressure or acidosis. ECF was also observed in the superfused papillary muscle in

which perfusion pressure is not involved in the contractile response.

Another factor which must be considered is the possibility that the removal of glucose could have a selective effect on membrane stability. This has been addressed previously in Chapter 4 but would be relevant here if this effect involved the early activation of K_{ATP}-channels. Both ischaemia and hypoxia have been associated with APD shortening (Vleugels *et al.*, 1980) although additional ionic currents may contribute to this effect. It was clear from the studies in the normoxic papillary muscles that the K_{ATP}-channel activator, cromakalim, shortened the ERP and this effect was sensitive to the KATP-channel inhibitor, glibenclamide. Although channel activation was not measured directly, this interaction supported the conclusion that the effects on ERP and contractile force induced by these compounds were the result of changes to K_{ATP}-channel activity. This showed that activation of the K_{ATP}-channel alone has a marked effect in cardiac tissue. Cromakalim caused ERP shortening and reduced contractile force which would support a role for K_{ATP}-channels in ECF. The apparent failure of glibenclamide to reverse the effects of cromakalim on contractile force could be explained by "run-down" during the duration of the experiments. The suggestion that the inneffectiveness of glibenclamide may be due to inhibition of calcium entry by glibenclamide (Bosmith *et al.*, 1989) is unlikely since glibenclamide had no effect on contractility either during pre-treatment or when superfused over longer durations in the present studies.

The effects of hypoxia in the papillary muscle preparation were similar to those of cromakalim in that ERP shortened and contractile force decreased. While glibenclamide reduced the effect of hypoxia on ERP, this was not fully prevented indicating that factors other than K_{ATP}-dependent channel activation may be involved in this response. Nevertheless, the results showed that a major component of hypoxia-induced ERP shortening does involve activation of these channels.

The effects of glibenclamide on the contractile response to hypoxia were more complex. Glibenclamide did not prevent the initial rapid failure of contractile force caused by hypoxia but did have a significant protective effect at later stages of hypoxia. This did not appear to involve changes in ATP content. In the normoxic papillary muscle, pre-treatment with glibenclamide completely inhibited the effects of subsequent superfusion with cromakalim. Since glibenclamide was present before hypoxia was introduced, it could be assumed that any effect of K_{ATP}-dependent channel activation as a result of hypoxia would also be completely inhibited. This was supported by the marked reduction of ERP shortening during hypoxia in the presence of glibenclamide. However, no reduction of the ECF response was observed. Despite the finding that glibenclamide improved contractile force later in the hypoxic period, the results suggested that ECF involves mechanisms other than K_{ATP}-dependent channel activation and cannot be directly linked to changes in action potential duration .

CHAPTER SEVEN

EPILOGUE: THE POTENTIAL OF METABOLIC INTERVENTION FOR FUTURE DRUG DEVELOPMENT

7.1 The role of metabolic substrates in cardiac ischaemia

A first objective of this thesis has been the development of the guinea-pig perfused heart model for the investigation of cardiac ischaemia. The guinea-pig was chosen because previous work had shown that the myocardium of this species compared more closely to human while rat heart exhibited noteable differences in calcium handling and action potential configuration (Clarke and Patmore, 1984) and in receptor profile (Flynn *et al.*, 1978; Steinforth *et al.*, 1992). In this thesis, the guinea-pig model has been shown to be responsive to both hypoxia and ischaemia and proved a suitable experimental system for investigations into the aspects of myocardial substrate utilisation.

The normoxic myocardium uses both glucose and fatty acids as the major substrates for ATP production and preferentially metabolises fatty acids when these are available. However, during ischaemia, impairment of the metabolism of either substrate has important implications for the subsequent recovery of the heart. In the studies presented in this thesis, the deleterious consequences of ischaemia (e.g. ATP depletion, contracture development, enzyme release) were increased when the heart was deprived of glucose or when fatty acid was present in addition to glucose. This showed directly that substrate availability could influence the consequences of ischaemia and indicated that promotion of glucose utilisation in preference to fatty acids may be beneficial during ischaemia.

Contracture development appears to be characteristic of conditions in which glucose metabolism is limited. In the studies carried out, it was common to both glucose-free low-flow ischaemia and hypoxia and to no-flow ischaemia. Interventions which prevent the development of contracture could offer advantages for reperfusion therapy because of the increased resistance to coronary flow (the *no-reflow* phenomenon) associated with contracture (Eberli *et al.*, 1991). It has been shown both in this thesis and by Owen *et al.*, (1990), that glucose metabolism can prevent or at least delay the onset of ischaemic contracture. Glucose-insulin therapy also markedly increases reperfusion recovery (Eberli *et al.*, 1991). The promotion

of glucose metabolism may help prevent the development of contracture perhaps by increasing cytosolic ATP.

Potassium loss is also a common feature of ischaemia, but the mechanism of activation and closure of the K_{ATP}-dependent channel and the contribution of this channel to other ischaemic events (e.g. early contractile failure) have been less well characterised. The majority of research into the effects of K_{ATP}-channel modulation has been directed towards activation of the channel as a mechanism of reducing ischaemic injury (Escande and Cavero, 1992). Cromakalim has a selective action on smooth muscle in comparison to heart (Quast and Cook, 1989) and vasodilatation of coronary vessels is likely to be a major factor in the protective action of these compounds. However, Auchampach *et al.* (1991) have reported that cardioprotection occurs in the absence of significant haemodynamic changes and have suggested that the protective effects of K_{ATP}-channel activators may result from a direct effect on the myocytes. Cellular K^+ loss and extracellular K^+ accummulation have been closely linked to the generation of ventricular arrhythmias during myocardial ischaemia, therefore, increased K_{ATP}-channel activation could precipitate or lower the threshold for arrhythmias.

The association of these channels with cardiac arrhythmia has also encouraged investigations into the effects of blocking K_{ATP}-channel activation during hypoxia and ischaemia. Glibenclamide has been shown to reduce K^+ loss and to have potential anti-arrhythmic effects in isolated rat hearts (Kantor *et al.*, 1990), in dogs in vivo (Bekheit et al., 1990) and in rabbit papillary muscles (Hicks and Cobbe, 1991). While these effects of glibenclamide may be beneficial, the involvement of K_{ATP}-channel activation in hypoxic and ischaemic dilation of coronary arteries (Daut *et al.*, 1990) may oppose the use of glibenclamide under these conditions.

As with contracture development, glucose metabolism has an important role in K_{ATP}-channel activity and it has been reported that glycolytic ATP preferentially inhibits K_{ATP}-channel activation in cardiac myocytes (Weiss and Lamp, 1987).

These findings again suggest that the key intervention during ischaemia could be increased glycolytic flux provided that sufficient stimulation of the pathway is possible.

7.2 Strategies to alter cardiac metabolism during ischaemia

Since the initial hypothesis of the glucose-fatty acid cycle, put forward by Randle in 1963, much has been learned about the regulation of glucose and fatty acid metabolism under normoxic and ischaemic conditions. Yet, clinical success in protecting the ischaemic heart by a selective action on substrate metabolism has been limited. With the exception of directly increasing the concentration of exogenous glucose (and insulin), the mechanisms through which myocardial glucose utilisation can be increased are limited.

7.2.1 Targetting fatty acid metabolism

Glucose metabolism can be increased by reducing fatty acid oxidation. This approach has two important consequences for the ischaemic heart. Glucose oxidation is increased and the potential deleterious effects of impaired fatty acid metabolism are diminished. A selective action targetted at ischaemic cells would represent an extremely effective method of achieving ischaemic protection. The inhibition of fatty acid metabolism during ischaemia is known to result in the accummulation of both fatty acyl-CoA and long-chain acylcarnitine (LCA) which may increase cell damage (Katz and Messineo, 1981; Corr *et al.*, 1981). In addition, the influence of fatty acids on glucose metabolism during ischaemia and reperfusion can profoundly affect the consequences of ischaemia in the heart (Lopashuk *et al.*, 1990). It is a widely accepted concept that reduction of fatty acid metabolism would be beneficial during ischaemia, however success in demonstrating the clinical effectiveness of this intervention has been limited

Initial studies were aimed at reducing lipolysis using nicotinic acid derivatives. It may be more important to inhibit lipolysis rather than fatty acid uptake alone, because lipolysis of endogenous triglycerides within the myocardium can contribute significantly to fatty acid metabolism (Saddik and Lopaschuk, 1992). Nicotinic acid inhibits lipolysis of triglycerides in adipose tissue (Vik-Mo *et al.,* 1986), but although analogues of nicotinic acid have been shown to be beneficial in experimental cardiac ischaemia (Vik-Mo *et al.*, 1986; van Bilsen *et al.*, 1990), their clinical usefulness is restricted because of adverse side-effects such as nausea, gastric irritation and possible hypotension. In addition, withdrawal from this type of drug results in rebound elevation of arterial fatty acids to concentrations in excess of the pre-treatment level (Vik-Mo and Mjos, 1981).

Another approach has been inhibition of the CPT-1 enzyme and reduction of long-chain fatty acid oxidation. Methyl-2-tetraglycidic acid was shown to be effective in increasing carbohydrate utilisation in diabetic patients (Tutwiler *et al.*, 1978). Further development led to the discovery of etomoxir (Wolf *et al.*, 1981) and although the CPT-1 inhibitors were initially aimed at improving glucose homeostasis in diabetes, it was also realised that this mechanism may have a potential use in reducing fatty acid metabolism in the ischaemic heart. The beneficial effects of CPT-I inhibition during ischaemia (Lopashuk *et al.*, 1990) may be related to the ability of these drugs to reduce both LCA accummulation (Paulson *et al.*, 1986) and myocardial oxygen consumption which is increased in the presence of fatty acids (Mjos, 1971). While these effects would be of limited value under conditions of no-flow ischaemia, they may be of importance under low-flow conditions where residual substrate and oxygen supply remain.

However, at the present time, CPT-1 inhibition, although effective in reducing fatty acid metabolism, has also failed to produce a successful clinical application. One reason for this may be that irreversible inhibition of the CPT-I enzyme and long-term inhibition of fatty acid metabolism may be deleterious to the heart and other tissues (Selby and Sherratt, 1989). Such an effect has been described for

oxfenicine which has CPT-1 inhibitory activity (Bielefeld *et al.*, 1985) and has been shown to produce cardiac hypertrophy (Greaves *et al.*, 1984). Although this may be an adaptive change in response to chronic inhibition of fatty acid metabolism, some other effect by oxfenicine itself cannot be excluded.

Secondary activation of PDH with subsequent stimulation of carbohydrate utilisation has also been reported for oxfenicine. This was due to inhibition of CPT-1 by 4-hydroxyphenyl-glyoxylate, a metabolite of oxfenicine (Higgins *et al.*, 1981). Oxfenicine demonstrated a cardio-selective action which represented an important development in this area. This property was due to greater oxfenicine transaminase activity in the heart and a higher degree of sensitivity of heart CPT-1 compared to liver (Stephens *et al.*, 1985). Two important properties are therefore required in the further development of this type of drug. Firstly, the CPT-1 inhibitor should be cardio-selective and secondly the inhibition should be reversible. Recently, a series of reversible CPT-1 inhibitors, indicated for use in cardiac ischaemia, have been patented by Hoffmann La Roche (European Patent Application EP 0 512 352 A2) and the clinical effectiveness of these compounds will be of great interest.

7.2.2 Targetting glucose metabolism

Another mechanism by which fatty acid metabolism could be reduced and glucose oxidation increased is through activation of pyruvate dehydrogenase (PDH, McAllister *et al.*, 1973; Whitehouse and Randle, 1973). A number of studies have shown that dichloracetate (DCA), an activator of PDH (Whitehouse and Randle, 1973), is associated with reduced myocardial ischaemic damage (Mjos *et al.*, 1976; Racey-Burns *et al.*, 1989; Lopashuk and Saddik, 1992). Although DCA has been shown to enhance myocardial efficiency and stimulate myocardial lactate utilisation in patients with coronary artery disease (Wargovich *et al.*, 1988), it has also been associated with potential toxicity and therefore clinical investigations have been

limited (Stacpoole, 1989).

Despite the overwhelming evidence that stimulation of glucose oxidation and inhibition of fatty acid oxidation are beneficial to the ischaemic myocardium (see summary table), at the present time no suitable drug has been identified which can successfully manipulate this "metabolic switch". The results described for ranolazine in this thesis, with regard to the protective effects associated with PDH activation, provide a possible mechanism by which this compound may reduce myocardial ischaemic damage. Clearly, however, further investigation is required to determine whether this is a direct effect on PDH or the consequence of some other interaction.

In conclusion, myocardial substrate regulation in the ischaemic myocardium is an area of research which offers much promise for future drug development.

SUMMARY OF THE EFFECTS OF SUBSTRATE UTILISATION IN THE ISCHAEMIC MYOCARDIUM

GLUCOSE	FATTY ACID
increases glycolytic ATP	decreases glycolytic ATP
maintains membrane stability	increases membrane dysfunction
inhibits K_{ATP} activation	K_{ATP} (?)
inhibits contracture development	increases contracture development
decreases fatty acid metabolism	increases oxygen consumption
	increases arrhythmia

The consequences of myocardial ischaemia can either be increased or reduced depending on the substrate metabolised. This summary table shows that promotion of glucose utilisation in favour of fatty acid is potentially beneficial to the ischaemic myocardium.

Bibliography

Adams, R. J., Cohen, D. W., Gupte, S., Johnston, D. J., Wallick, E. T., Wang, T. and Schwartz, A. (1979) J. Biol. Chem. **254**, 12404-12410

Aksnes, G. (1992) J. Mol. Cell. Cardiol. **24**, 323-331

Allely, M. C. and Alps, B. J. (1988) Br. J. Pharmacol. **93**, 246p

Allely, M. C. and Alps, B. J. (1989) Br. J. Pharmacol. **96**, 977-985

Allely, M. C., Alps, B. J. and Kilpatrick, A. T. (1987) Biochem. Soc. Trans. **15**, 1057-1058

Allen, D. G. (1977) Cardiovasc. Res. **11**, 210-218

Allen, D.G. and Orchard, C. H. (1983) J. Physiol. **339**, 107-122

Allen, D. G. and Orchard, C. H. (1987) Circ. Res. **60**. 153-168

Allen, D. G. and Smith, G. L. (1985) J. Physiol. (Lond). **369**, 92p

Allen, D. G., Morris, P. G., Orchard, C. H. and Pirolo, J. S. (1985) J. Physiol. **361**, 185-204

Arai, A. E., Pantely, G. A., Thomas, W. J., Anselone, C. G. and Bristow, J. D. (1992) Circ. Res. **70**, 1137-1145

Auchampach, J. A., Maruyama, M., Cavero, I. and Gross, G.J. (1991) J. Pharm. Exp. Ther. 259, 961-967

Ball, E.G. (1965) Ann. N. Y. Acad. Sci. **131**, 225-234

Barry, W. H., Peeters, G. A., Rasmussen, C. A. F. and Cunningham, M. J. (1987) Circ. Res. **61**, 726-734

Bekheit, S., Restivo, M., Boutjdir, M., Henkin, R., Gooyandeh, K., Assadi, M., Khatib, S., Gough, W.B. and El-Sherif, N. (1990) Am. H. J. **119**, 1025-1033

Belfrage, P., Fredrickson, G., Olsson, H. and Stralfors, P. (1984) Adv. Cyc. Nucl. Pro. Phos. Res. **17**, 351-359

Bersohn, M. M., Philipson, K. D. and Fukushima, J. Y. (1982) Am. J. Physiol. **242**, C288-C295

Bielfield, D. R., Vary, T. C. and Neely, J. R. (1985) J. Mol. Cell. Cardiol. **17**. 619-625

Bing, R. J. (1954) in The Metabolism of the Heart. Harvey Lecture Series. **50**, pp. 20-70, Academic Press. New York

Bing, O. H. L. and Fishbein, M. C. (1979) Circ. Res. **45**, 298-308

Bing, O. H. L., Brooks, W. W. and Messer, J. V. (1973) Science. **180**, 1297-1280

Bing, O. H. L., Apstein, C. S. and Brooks, W. W. (1975) in Recent Advances In Studies On Cardiac Structure And Metabolism (Roy, P.E. and Rona, G. eds.), **10**, pp. 343-354, University Park Press, Baltimore

Black, J. W. and Stephenson, J. S. (1962) Lancet. **1**, 311

Boddeke, E., Hugtenburg, J., Jap, W., Heynis, J. and van Zweiten, P. (1989) Trends. Pharmacol. Sci. **10**, 397-400

Bohmer, T., Rydning, A. and Solberg, H. E. (1974) Clinica. Chimica. Acta. **57**, 55-61

Borst, P., Loos, J. A., Christ, E.J. and Slater, E.C. (1962) Biochim. Biophys. Acta **62**, 509-518

Bosmith, R. E., Briggs, I., Grant, T. L., Grimwood, S., Russell, N. J., Stone, M. A. and Wickenden, A. D. (1989) Pfleugers. Arch. **414**, S190

Bourdillon, P. D. and Poole-Wilson, P. A. (1982) Circ. Res. **50**, 360-368

Bourdillon, P. D., Lorrell, B.H., Mirsky, I., Paulus, W. J., Wynne, J. and Grossman, W. (1983) Circulation. **67**, 316-323

Bowers, K.C., Allshire, A.P. and Cobbold, P.H. (1992) J. Mol. Cell. Cardiol. **24**, 213-218

Braveny, P. and Sumbera, J. (1970) Pflugers. Arch. **319**, 36-48

Bricknell, O. L. and Opie, H. L. (1978a) in Recent Advances In Studies On Cardiac Structure and Metabolism (Kobayashi, T., Sano, T. and Dhalla, N. S. eds.), pp. 509-518, University Park Press, Baltimore

Bricknell, O. L. and Opie, L. H. (1978b) Circ. Res. **43**, 102-115

Bricknell, O. L. and Opie, L. H. (1984) Cardiovasc. Res. **18**, 145-148

Bricknell, O. L, Daries, P. S. and Opie, L. H. (1981) J. Mol. Cell. Cardiol. **13**, 941-945

Brillantes, A. M., Allan, P., Takahashi, T., Izumo, S. and Marks, A. R. (1992) Circ. Res. **71**, 18-26

Brosnan, J. T. and Fitz, I. B. (1971) Biochem. J. **125**, 94p-95p

Brownsey, R. W. and Brundt, R. V. (1977) Clin. Sci. Mol. Med. **53**, 513-521

Brunton, T. L. (1867) Lancet. **ii**, 97

Bunger, R., Permanetter, B. and Yaffe, S. (1983) Pfleugers. Arch. **397**, 214-219

Chock, P. B., Rhee, S. G. and Stadtman, E. R. (1980) Ann. Rev. Biochem. **49**, 813-843

Carlstein, A., Hallgren, B., Jagenburg, R., Svanborg, A. and Werko, L. (1961) Scand. J. Clin. Lab. Invest. **13**, 418-428

Carmeliet, E. (1978) Circ. Res. **42**, 577-587

Caroni, P. and Carafoli, E. (1981) J. Biol. Chem. **256**, 3263-3270

Caroni, P. and Carafoli, E. (1983) Eur. J. Biochem. **132**, 451-460

Challoner, D. R. and Steinberg, D. (1966a) Am. J. Physiol. **210**, 280-286

Challoner, D. R. and Steinberg, D. (1966b) Am. J. Physiol. **211**, 897-902

Christian, D. R., Kilshmeir, G. S., Pettett, G., Paradise, R. and Ashmore, J. (1969) Adv. Enz. Reg. **7**, 71-81

Clark, M. G. and Patten, G. S. (1984) J. Biol. Chem. **259**, 15204-15211

Clarke, B. and Patmore, L. (1984) Br. J. Pharmacol. **83**, 438p

Clarke, B., O'Connor, J., Duncan, G. P., Patmore, L. and Spedding, M. (1990) J. Mol. Cell. Cardiol. **22**, (Suppl III), S112

Cobbold, P. H. and Bourne, P. K. (1984) Nature. **312**, 444-446

Corr, P. B., Snyder, D. W., Cain, M. E., Crafford, W. A., Gross, R. W. and Sobel, B. E. (1981) Circ. Res. **49**, 354-363

Corr, P. B., Snyder, D. W., Lee, B. I., Gross, R. W., Kein, C. R. and Sobel, B. E. (1982) Am. J. Physiol. **12**, H187-H195

Corr, P.B., Gross, R.W. and Sobel, B.E. (1984) Circ. Res. **55**, 135-154

Cowan, J. C. and Vaughan Williams, E. M. (1980) J. Mol. Cell. Cardiol. **12**, 347-369

Crass, M. F. (1972) Biochim. Biophys. Acta. **280**, 71-81

Daut, J., Maier-Rudolph, W., Beckerath, N., Mehrke, G., Gunther, K. and Goedel-Meinen, L. (1990) Science. **247**, 1341-1344

Davies, M. J. (1990) Circulation. **82** (Suppl II), II38-II46

Davis, J. A. (1955). Am. J. Med. Sci. **230**, 259-263.

Dawson, M. J., Gadian, D. G. and Wilkie, D. R. (1980) J. Physiol. **299**, 465-484

de Leiris, J. and Feuvray, D. (1977) J. Mol. Cell. Cardiol. **9**, 365-373

de Leiris, J. and Opie, L. H. (1978) Cardiovasc. Res. **12**, 585-596

Dennis, S. C., Hearse, D. J. and Coltart, D. J. (1982) Cardiov. Res. 16. 209-219

d'Hemecourt, A. and Detour, R. (1978) in Primary and Secondary Angina Pectoris (Maseri, A., Klasen, G. A. and Lesch, M., eds.), pp. 177 - 185. Grune and Stratton Inc

Doorey, A. J. and Barry, W. H. (1983) Circ. Res. **53**, 192-201

Downar, E., Janse, M. J. and Durrer, D. (1977) Circulation. **56**, 217-224

Eberli, F. R., Weinberg, E. O., Grice, W..N., Horowitz, G. L. and Apstein, C. S. (1991) Circ. Res. **68**, 466-481

Elliot, A. C., Smith, G. L. and Allen, D. G. (1989) Circ. Res. **64**, 583-591

Ellis, D. and Thomas, R. C. (1976) J. Physiol. **262**, 755-771

Escande, D., Thuringer, D., Leguern, S. and Cavero, I. (1988) Biochem. Biophys. Res. Commun. **154**, 620-625

Escande, D. and Cavero, I. (1992) Trends. Pharmacol. Sci.. **13**, 269-272

Evans, J. R., Opie, L. H. and Shipp, J. C. (1963) Am. J. Physiol. **205**, 766-770

Farah, A. E. and Alousi, A. A. (1981) Life. Sci. **29**, 975-1000

Fawaz, G., Hawa, E. S. and Turanji, B. (1957) Br. J. Pharmacol. **12**, 270-279

Feldman, R.L. (1987) Am. J. Cardiol. **59**, 1187-1190

Ferrandon, P., Pascal, J. C. and Armstrong, J.M. (1988) Br. J. Pharmacol. **93**, 247p

Ferrandon, P., Chaylat, C., Fliegel, E. and Armstrong, J. M. (1990) Fund. Clin. Pharmacol. **4**, 426

Ferrandon, P., Chaylat, C., Michel, D. and Armstrong, J. M. (1992) J. Mol. Cell. Cardiol. **24** (Suppl IV), S56

Feuvray, D. (1981) Am. J. Physiol. **240**, H391-398

Feuvray, D. and Plouet, N. (1981) Circ. Res. **48**, 740-747

Flaherty, J. T., Weisfeldt, M., Buckley, B. H., Gardner, T. J., Gott, V. L. and Jacobus, W. (1982) Circulation. **65**, 561-571

Fleckenstein, A. (1977) Ann. Rev. Pharmacol. Toxicol. **17**, 149-166

Flynn, S. B., Gristwood, R. W. and Owen, D. A. A. (1978) J. Pharmacol. Methods. **1**. 183-195

Forssman, O., Hansonn, G. and Jensen. C. C. (1952) Acta. Med. Scand. **142**, 441-449

Fournier, N. C., Zuker, M., Williams, R. E. and Smith, I. C. P. (1983) Biochemistry. **22**, 1863-1872

Fournier, N. C. and Rahim, M. (1985) Biochemistry. **24**, 2387-2396

Fowler, N. O. (1971) Circulation. **44**, 755

Frank, J. S., Rich, T. L., Beydler, S. and Kreman, M. (1982) Circ. Res. **51**, 117-130

Fredrickson, D. S. and Gordon, R. S. (1958) Physiol. Rev. **38**, 585-630

Ganote, C. E. and Kaltenbach, J. P. (1979) J. Mol. Cell. Cardiol. **11**, 389-406

Ganote, C. E., Liu, S. Y., Safavi, S. and Kaltenbach, J. P. (1981) J. Mol. Cell. Cardiol. **13**, 93-106

Garlin, R., Fuster, V., Ambrose, J.A. (1986) Circulation. **74**, 6-9

Gevers, W. (1977) J. Mol. Cell. Cardiol. **9**, 867-874

Ghitescu, L., Fixman, A., Simionescu, M. and Simionescu, N. (1986) J. Cell. Biol. **102**, 1304-1311

Goldberg, D. I. and Khoo, J. C. (1985) J. Biol. Chem. **260**, 5879-5882

Greaves, P., Martin, J., Michel, M. C. and Mompon, P. (1984) Arch. Toxicol. **7**, 488-493

Groot, P. H. E., Scholte, H. R. and Hulsmann, W. C.(1976) Adv. Lipid. Res. **14**, 75-126

Grover, F. L., Fewel, J. G., Ghidoni, J. J. and Trinkle, J. K. (1981) J. Thorac. Cardiovasc. Surg. **81**, 11-20

Gruber, W. (1978) Clin. Chem. **24**, 177

Gudbjarson, S., Mathers, P. and Ravens, K. G. (1970) J. Mol. Cell. Cardiol. 1, 325-339

Haddock, B. A., Yates, D. W. and Garland, P. B. (1970) Biochem. J. **119**, 565-573

Hansford, R. G., Hogue, B., Prokopczuk, A., Wasilewska, E and Lewartowski, B. (1990) Biochim. Biophys. Acta. **1018**, 282-286

Harding, D. P. and Poole-Wilson, P. A. (1980) Cardiovasc. Res. **14**, 435-445

Hearse, D. J. (1977) J. Mol. Med. **2**, 185-200

Hearse, D. J. (1979) Am. J. Cardiol. **44**, 1115-1121

Hearse, D. J. (1980) J. Physiol (Paris). **76**, 751-768

Hearse, D. J. and Chain, E. B. (1972) Biochem. J. **128**, 1125-1133

Hearse, D. J., Garlick, P. B. and Humphrey, J. M. (1977) Am. J. Cardiol. 39, 986-993

Hearse, D. J., Humphrey, S. M., Boink, A. B. T. J. and Ruigrok, T. J. C. (1978) Eur. J. Cardiol. 7, 241-256

Heathers, G. P. and Brundt, R. V. (1985) J. Mol. Cell. Cardiol. **17**, 907-916

Heberden, W. (1772) M. Trans. Roy. Coll. Physicians (Lond). **2**, 59-67

Henderson, A. H., Most, A. S., Parmley, W. W., Gorlin, R. and Sonnenblick, E. H. (1970) Circ. Res. **26**, 439-449

Herrick, J. B. (1912) J. Am. Med. Assoc. **59**, 2015

Hewitt, R. L., Lolley, D. M., Adrouny, G. A. and Drapanas. T. (1973) Surgery. **73**, 444-453

Hicks, M. N. and Cobbe, S. M. (1991) Cardiovasc. Res. **25**, 407-413

Higgins, A. J., Morville, M., Burges, R. A., Gardiner, D. G., Page, M. G. and Blackburn, K. J. (1980) Life Sci. **27**, 963-970.

Higgins, A.J., Morville, M., Burges, R. A. and Blackburn, K. J. (1981) Biochem. Biophys. Res. Comm. **100**, 291-269

Hillis, D. L. and Braunwald, E. (1977) N. Eng. J. Med. **296**, 1034-1041

Hollman, A. (1963) Med. World. **99**, 217

Holubarsch, Ch., Alport, N. R., Goulette, R. and Mulieri, L. A. (1982) Circ. Res. **51**, 777-786

Hough, F. S. and Gevers, W. (1975) South. Afr. Med. J. **49**, 538-543

Hutter, J. F. and Sobol, S. (1992) Int. J. Biochem. **24**, 399-403

Ichihara, K., Sakai, K. and Abiko, Y. (1986) Arch. Int. Pharmacodyn. **280**, 58-73

Idell-Wenger, J. A., Grotyohann, L. W. and Neely, J. R. (1978) J. Biol. Chem. **253**, 4310-4318

Inoue, D and Pappano, A. J. (1983) Circ. Res. **52**, 625-634

Irisawa, H. and Kokubun, S. (1983) J. Physiol (Lond). **338**, 321-338

Janse, M. J. and Kleber, A. G. (1981) Circ. Res. **49**, 1069-1081

Jedicken, L. A. (1964) Circ. Res. **14**, 202-211

Jennings, R. B. and Ganote, C. E. (1974) Circ. Res. **35** (Suppl III), 156

Jennings, R.B., Murry, C. E., Steenbergen, C. and Reimer, K. A. (1990) Circulation. **82** (Suppl II), II 2-II 12

Jodalen, H., Stangeland, L., Grong, K., Vik-Mo, H. and Lekven, J. (1985) J. Mol. Cell. Cardiol. **17**, 973-980

Jugdutt, B. I., Hutchins, G.M., Bulkley, B.H. and Becher, L. C. (1979) Circulation. **60**, 1141-1150

Julian, D. G. (1982) in What is Angina (Julian, D. G., Lie, K.I. and Wilhelmsen, L., eds.) pp. 12-13, A.B.Hasle, Molndal, Sweden

Kantor, P. F., Coetzee, W. A., Carmeliet, E. E., Dennis S.C. and Opie L.H. (1990) Circ. Res. **66**. 478-485

Kaplan, P., Hendrikx, M., Mattheusen, M., Mubagura, K. and Fleming, W. (1992) Circ. Res. **71**, 1123-1130

Karwatowska-Krynska, E. and Beresewicz, A. (1983) J. Mol. Cell. Cardiol. **15**, 523-536

Katz, A. M. (1992) Physiology of the Heart. (2nd Edition). Raven Press, New York

Katz, A. M.(1973) Am. J. Cardiol. **32**, 456-460

Katz, A. M. and Messineo, F. C. (1981) Circ. Res. **48**, 1-16

Kavaler, F., Hyman, P. M. and Lefkowitz, R. B. (1972) J. Gen. Physiol. **60**, 351- 365

Kawachi, Y., Tomoike, H., Maruoka, Y., Kikuchi, Y., Araki, H., Ishii, Y., Tanaka, K. and Nakamura, M. (1984) Circulation. **69**, 440 -450

Kennedy, E. P. (1961) Fed. Proc. **20**, 934-940

Khan, M. H. (1926) Am. J. Med. Sci. **172**, 418

Kholhardt, M. and Kubler, M. (1975) Naunyn-Schmiedebergs. Arch. Pharmacol. **290**, 265-274

Kihara, Y., Grossman, W. and Morgan, J. P. (1989) Circ. Res. **65**, 1029-1044

Kjekshus, J. K. and Mjos, O. D. (1972) J. Clin. Invest. **51**, 1767-1776

Klein, M. S., Goldstein, R. A., Welch, M. J. and Sobel, B. E. (1979) Am. J. Physiol. **237**, H51-H57

Knabb, M. T., Saffitz, J. E., Corr, P. B. and Sobel, B. E. (1986) Circ. Res. **58**, 230-240

Kobayashi, K. and Neely, J. R. (1979) Circ. Res. **44**, 166-175

Kobayashi, K. and Neely, J. R. (1983) J. Mol. Cell. Cardiol. **15**, 359-367

Kohlhardt, M., Haap, K. and Figulla, H. R. (1976) Pflugers. Arch. **366**, 31-38

Koretsune, Y. and Marban, E. (1990) Circulation. **82**, 528-535

Krause, S. M. and Hess, M. L. (1984) Circ. Res. **55**, 176-184

Krause, S. M., Jacobus, W. E. and Becker, L. C. (1989) Circ. Res. **65**, 526-530

Kubler, W. and Spiekerman, P. G. (1970) J. Mol. Cell. Cardiol. **1**, 351-377

Kurien, V. A. and Oliver, M. F. (1970) Lancet. **1**, 813-815

Langendorff, O. (1895) Pfleugers. Arch. **61**, 291-332

La Noue, K. F. and Schoolworth, A. C. (1979) Ann. Rev. Biochem. **48**. 871-992

Larsen, T. S., Myrmel, T., Skulberg, A., Severson, D. L. and Mjos, O. D. (1989) Mol. Cell. Biochem. **88**, 139-144

Lazdunski, M., Frelin, C. and Vigne, P. (1985) J. Mol. Cell. Cardiol. **17**, 1029-1042

Lee, H. C., Smith, N., Mohabir, R. and Clusin, W. T. (1987) Proc. Natl. Acad. Sci. **84**, 7793-7796

Liedtke, A, J. (1981) Prog. Cardiovasc. Dis. **23**, 321-336

Liedtke, A. J., Nellis, S. and Neely, J. R. (1978) Circ. Res. **43**, 652-661

Lipasti, J. A., Nevalaimen, T. J., Alanen, K. A. and Tolvanen, M. A. (1978) Circ. Res. **43**, 102-115

Little, S. E., Van der Vusse, G. J., Bassingthwaite, J. B.(1986) J. Nucl. Med. **27**, 966

Lodge, N. J. and Gelband, H. (1988) Cardiovasc. Res. **22**, 520-526

Lolley, D. M., Ray, J. F., Myers, W. O., Sautter, R. D. and Tewksbury, D. A. (1979) J. Thorac. Cardiovasc. Surg. **78**, 678-687

Lopaschuk, G. D. and Saddik, M. (1992) Mol. Cell. Biochem. **21**, 111-116

Lopaschuk, G.D., Wall, S. R., Olley, P. M. and Davies. N. J. (1988) Circ. Res. **63**, 1036-1043

Lopaschuk, G.D., Spafford, M.A., Davies, N.J. and Wall, S.R. (1990) Circ. Res. **66**, 546-553

Lott, J. A. and Stang, J. M. (1980) Clin. Chem. **26**, 1241-1250

MacLeod, D. P and Daniel, E. E. (1965) J. Gen. Physiol. **48**, 887-899

MacLeod, D. P and Prasad, K. (1969) J. Gen. Physiol. **53**, 792-815

Malloy, C. R., Matthews, P. M., Smith, M. B., Radda, G. K. (1986) Cardiovasc. Res. **20**, 710-720

Mandel, F., Kranias, E. G., Grassi de Gerde, F., Sumida, A. and Schwartz, A. (1982) Circ. Res. **50**, 310-317

Marban, E., Kitzakaze, M., Kusuoka, H., Porterfield, J. K., Yue, D. T. and Chacko, V. P. (1987) Proc. Natl. Acad. Sci. USA. **84**, 6005-6009

Marzilli, M., Maseri, A., L'Abbate, A., Michaelassi, C. and Pisani, P. (1978) in Primary and Secondary angina pectoris (Maseri, A., Klassen, G.A. and Lesch. M., eds.), pp. 83-93, Grune and Stratton Inc, NY

Maseri, A., L'Abbate, A., Boroldi, G., Chierchia, S. and Margilli, M. (1978) New. Eng. J. Med. **299** 1271-1277

Matthews, P. M., Radda, G. K. and Taylor, D. J. (1981) Biochem. Soc. Trans. **9**, 236-237

McAllister, A., Allison, S. P. and Randle, P. J. (1973) Biochem. J. **134**, 1067-1081

McCallister, L. P., Daiello, A. C. and Tyers, G. F. O. (1978) J. Mol. Cell. Cardiol. **10**, 67-80

McCormack, J. G. and Denton, R. M. (1984) Biochem. J. **218**, 235-247

McCormack, J. G. and Denton, R. M. (1989) Methods. Enzymol. **174**, 95-118

McDonald, T. F. and MacLeod, D. P. (1973) J. Physiol. **229**, 559-582

McLarin, L. P., Rollett, E. L. and Grossman, W. (1973) Am. J. Cardiol. **32**, 751-757

Meller, J., Pichard, A. and Dack, S. (1976) Am. J. Cardiol. **37**, 938-940

Messineo, F. C., Pinto, P. B. and Katz, A. M. (1982) Adv. Myocardiol. **3**, 407-415

Mjos, O.D. (1971) J. Clin. Invest. **50**, 1386-1389

Mjos, O. D. and Kjekshus, J. K. (1971) Scand. J. Clin. Invest. **28**, 389-393

Mjos, O. D., Kjekshus, J. K. and Lekvan, J. (1974) J. Clin. Invest. **53**, 1290-1299

Mjos, O. D., Miller, N. E., Riemersma, R. A. and Oliver, M. F. (1976) Cardiovasc. Res. **10**, 427-436

Momomura, S., Ingwall, J. S., Parker, J. A., Sahagian, P., Ferguson, J. J. and Grossman, W. (1985) Circ. Res. **57**, 822-835

Moore, K.H. (1985) Mol. Physiol. **8**, 549-563

Moore, K. H., Bonema, J. D. and Solomon, F. J. (1984) J. Mol. Cell. Cardiol. **16**, 905-913

Morad, M. and Goldman, Y. (1973) Prog. Biophys. Mol. Biol. **27**, 257-313

Moran, N. C. and Perkins, M. E. (1958) J. Pharm. Exp. Ther. **124**, 223-233

Moreau, D. and Chardigny, J. M. (1991) J. Mol. Cell. Cardiol. **23**, 1165-1176

Morrisett. J. D., Powanall. H. J., Gotto, A. M. (1975) J. Biol. Chem. **250**, 24-87

Movesesian, M. A., Bristow, M. R. and Krall, J. (1989) Circ. Res. **65**, 1141-1144

Mullins, L. I. (1979) Am. J. Physiol. **236**, C103-C110

Myrmel, T., Forsdahl, K., Sager, G. and Larsen, T. S. (1991) J. Mol. Cell. Cardiol. **23**, 207-215

Nakamura, S., Kiyosue, T. and Arita, M. (1989) Cardiovasc. Res. **23**, 286-294

Nayler, W. G., Yepez, C. E. and Poole-Wilson, P. A. (1978) Cardiovasc. Res. **12**, 666-674

Nayler, W. G., Poole-Wilson, P. A. and Williams, A. (1979) J. Mol. Cell. Cardiol. **11**, 683-706

Nayler, W. G., Buckley, D. J. and Elz, J. S. (1988) in Diastolic relaxation of the heart (Grossman, W. and Lorrell, B. H., eds.), pp. 67-72. Martinus Nijhoff

Neely, J. R. and Morgan, H.E. (1974) Ann. Rev. Physiol. **36**, 413-459

Neely, J. R., Rovetto, M. J., Whitmer, J. R. and Morgan, H.E. (1973) Am. J. Physiol. **225**, 651-658

Neely, J. R., Garber, D., McDonough, K. and Idell-Wenger, J. (1979) in Perspective in Cardiovascascular Research (Winbury, M. M. and Abiko, Y., eds.), **3**, pp. 225-239, Raven Press, New York

Neill, W. A., Krasnow, N., Levine, H. J. and Gorlin, R. (1963) Am. J. Physiol. **204**, 427-432

Nichols, C. G., Ripoli, C. and Lederer, W. J. (1991) Circ. Res. **68**, 280-287

Noll, F. (1974) in Methods of Enzymatic Analysis, 2nd Edition (Bergemeyer, H. U., ed.), pp. 1475, Academic Press, London

Noma, A. (1983) Nature. **305**, 147-148

Noma, A. and Shibasaka, T. (1985) J. Physiol (Lond). **363**, 463-480

Northover, B. J. (1991) Br. J. Pharmacol. **103**, 1225-1229

Oliver, M. F., Kurien, V. A. and Greenwood, T. W. (1968) Lancet. **1**, 710-715

Olson, R. E. and Hoeschen, R. J. (1967) Biochem. J. **103**, 796-801

Otani, H., Tanaka, H., Inoue, T., Umemeto, M., Omoto, K., Tanaka, K., Sato, T., Osako, T., Masuda, A., Nonoyama, A. and Kagawa, T. (1984) Circ. Res. **55**, 168-175

Opie, L. H. (1968) Am. Heart. J. **76**, 685-698

Opie, L. H. (1975) Am. J. Cardiol. **36**, 938-953

Opie, L. H. (1976) Circ. Res. **38**, 52-74

Opie, L. H. (1979) Am. Heart. J. **97**, 375-386

Opie, L. H. (1991) The Heart (2nd Edition). Raven Press. New York

Opie, L. H. (1992) Cardiovasc. Res. **26**, 721-733

Opie, L. H. and Bricknell, O. L. (1979) Cardiovasc. Res. 13, 693-702

Opie, L. H. and de Leiris, J. (1979) in Enzymes in Cardiology Diagnosis and Research (Hearse, D. J. and de Leiris, J., eds.), pp. 481-502, John Wiley & Sons, Chichester

Opie, L. H., Norris, R. M., Thomas, M., Holland, A. J., Owen, P. and Van Noorden, S. (1971) Lancet. **1**, 818-822

Oram, J. F., Bennetch, S. L. and Neely, J. R. (1973) J. Biol. Chem. **248**, 5299-5309

Orsonio-Vargas, A. R., Berezesky, I. K. and Trump, B. F. (1981) Scan. Electron. Micro. **2**, 463-472

Owen, P., Dennis, S. and Opie, L. H. (1990) Circ. Res. **66**, 344-354

Owens, K., Kennett, F. F. and Weglicki, W. B. (1982) Am. J. Physiol. **242**, H456-H461

Packer, M. (1989) New. Eng. J. Med. **320**, 709-718

Pande, S. V. (1975) Proc. Natl. Acad. Sci. **72**, 883-887

Pande, S. V. and Blanchaer, M. C. (1971) J. Biol. Chem. **246**, 402-411

Patel, T. B. and Olson, M. S. (1984) Am. J. Physiol. **246**, H858-H864

Paulson, D. J., Schmidt, M. J., Romens, J. and Shug, A. L. (1984) Bas. Res. Cardiol. **79**, 551-561

Paulson, D. J., Noonan, J. J., Ward, K. M., Stanley, H., Sherratt, A. and Shug, A. L. (1986) Basic. Res. Cardiol. **81**, 180-187

Pauly, D. F., Kirk, K. A. and MacMillin, J. B. (1991) Circ. Res. 68, 1085-1094

Pearson, D. J., Tubbs, P. K. and Chase, J. F. A. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.) pp. 1769, Academic Press. USA

Pieper, G. M., Todd, G. L., Shao, T. W., Salhany, J. M., Clayton, F. C. and Eliot, R. S. (1980) Cardiovasc. Res. **14**, 646-653

Piper, H.M. and Das, A. (1986) Basic. Res. Cardiol. **81**, 373-383

Piper, H. M., Sezer, O., Schwartz, P., Hutter, J. F. and Spiekermann, P.G. (1983) Biochim. Biophys. Acta. **732**, 193-203

Pitts, B. J. R., Tate, C. A., van Winkle, W. B., Wood, J. M. and Entman, M. L. (1978) Life. Sci. **23**, 391-402

Podzuweit, T., Dalby, A. J., Cherry, G. W. and Opie, L. H. (1978) J. Mol. Cell. Cardiol. **10**, 81-94

Pool, P. E., Cavell, J. W., Chidsey, C. A. and Braunwald, E. G. (1966) Circ. Res. **19**, 221-229

Prinzen, F. W., van der Vusse, G. J., Arts, T., Roemen, T. H. M., Coumans, W. A. and Reneman, R. S. (1984) Am. J. Physiol. **247**, H264-H272

Prinzmetal, M., Kennamer, R., Merliss, R., Wada, T. and Bor, N. (1959) Am. J. Med. **27**, 375-388

Quast, U. and Cook, N.S. (1989) Trends. Pharmacol. Sci.. **10**, 431-435

Raab, W. (1963) Am. Heart. J. **66**, 685-706

Racey-Burns, L. A., Burns, A. H., Summer, W. R. and Shepherd, R. E. (1989) Life Sci. **44**, 2015-2023

Ramsey, R. R. and Tubbs, P. K. (1975) FEBS. Lett. **54**, 21-25

Randle, P. J (1963) Ann. Rev. Physiol. **25**, 291-321

Randle, P. J. (1986) Biochem. Soc. Trans. **14**, 799-806

Randle, P. J., Garland, P. B., Hales, C. N. and Newsholme, E. A. (1963) Lancet. **1**, 785-789

Rauch, B., Bode, Ch., Piper, H. M., Hutter, J. F., Zimmerman, R., Braunwell, E., Hasselbach, W. and Kubler, W. (1987) J. Mol. Cell. Cardiol. **19**, 159-166

Renstrom, B., Nellis, S.H. and Liedtke. A.J. (1990) Circ. Res. **66**, 282-288

Ricciuti, M. A. (1972) Am. J. Cardiol. **30**, 498-502

Rich, T. L. and Langer, G. A. (1982) Circ. Res. **51**, 131-141

Rivas, F., Cobb, F.R., Bache, R.J. and Greenfield, J.C. (1976) Circ. Res. **38**, 439-447

Rovetto, M. J., Whitmer, J. T. and Neely, J. R. (1973) Circ. Res. **32**, 699-711

Rovetto, M. J., Whitmer, J. T. and Neely, J. R. (1975) Circ Res. **37**, 742-751

Russell, D.C. and Oliver, M.F. (1979). J. Mol .Cell. Cardiol. **11**, 31-44

Saddik, M. and Lopaschuk, G. D. (1991) J. Biol. Chem. **266**, 8162-8170

Saddik, M. and Lopaschuk, G. D. (1992) J. Biol. Chem. **267**, 3825-3831

Sakai, K. and Abiko, Y. (1981) Jpn. Circ. J. **45**, 1250-1255

Scheuer, J. and Brachfeld, N. (1966) Metabolism. **15**, 945-954

Scheuer, J. and Stezoski, S. W. (1970) Circ. Res. **27**, 835-849

Schmidt, F. H. (1961) Klin. Wschr. **39**, 1244

Schoonderwoerd, K., Broekhoven-Schokker, S., Hulsmann, W. C. and Stam, H. (1987) in Lipid metabolism in the normoxic and ischaemic heart (Stam, H. and van der Vusse, G. J., eds.), pp. 29-35, Springer-Verlag, New York

Schoutsen, R., Blom, J. J., Verdouw, P. D. and Lamers, J. M. (1989) J. Mol. Cell. Cardiol. **21**, 719-727

Schramm, M., Thomas, G., Towart, R. and Frankowiak, G. (1983) Nature. **303**, 535-537

Seamon, K. B. and Daly, J. W. (1983) Trends. Pharmacol. Sci.. **4**, 120-123

Selby, P. L. and Sherratt, S. A. (1989) Trends. Pharmacol. Sci.. **10**, 495-500

Sellke, F. W., Armstrong, M. L. and Harrison, D. G. (1990) Circulation. **81**, 1586-1593

Severson, D. L. and Hurley, B. (1982) J. Mol. Cell. Cardiol. **14**, 467-474

Shipp, J. C., Thomas, J. M. and Crevasse, L. (1964) Science. **143**, 371-373

Shrago, E. (1976) J. Mol. Cell. Cardiol. **8**, 497-500

Shrago, E., Shug, A. L., Sul, H., Bittar, N. and Folts, J. D. (1976) Circ. Res. **38** (Suppl 1), 75-78

Shug, A. L., Shrago, E., Bittar, N., Folts, J. D. and Koke, J. R. (1975) Am. J. Physiol. **228**, 689-692

Shug, A. L., Thomsen, J. H., Folts, J. D., Bittar, N., Klein, M. I., Koke, J. R. and Huth, P. J. (1978) Arch. Biochem. Biophys. **187**, 25-33

Simonsen, S. and Kjekshus, J. K. (1978) Circulation. **58**, 484-490

Singh, B. N., Collett, J. T. and Chow, C. Y. C. (1980) Prog. Cardiovasc. Disease. **22**, 243-301

Sleator, W., Furchgott, R. F., Gubareff, T. D. and Krespi, V. (1964) Am. J. Physiol. **206**, 270-282

Spector, A. (1971) Prog. Biochem. Pharmacol. **6**, 130-176

Stacpoole, P. W. (1989) Metab. Clin. Exper. **38**, 1124-1144

Stam, H. and Hulsmann, W. C. (1985) Eur. Heart. J. **6**, 158-167

Stam, H., Broekhoven-Schokker, S. and Hulsmann, W. C. (1986) Biochim. Biophys. Acta. **875**, 87-96

Stanfield, P. R. (1987) Trends. Neurol. Sci. **10**, 335-339

Steenbergen, C., Levy, L., Murphy, E. and London, R. E. (1987) Circ. Res. **60**, 700-707

Steenbergen, C., Murphy, E., Watts, J. A. and London, R. E. (1990) Circ. Res. **66**, 135-146

Steinforth, M., Chen, Y., Lavicky, J., Magnussen, O., Nose, M., Bossway, S., Schmitz, W. and Scholz, H. (1992) Br. J. Pharmacol. **107**, 185-188

Stephens, T. W., Higgins, A. J., Cook, G. A. and Harris, R. A. (1985) Biochem. J. **227**, 651-660

Stremmel, W., Strohmeyer, G., Borchard, F., Kowcha, S.and Berk, P. D. (1985) Proc. Natl. Acad. Soc. **82**, 4-8

Sumbera, J. (1970) Experentia. **26**, 738-739

Surer, J. R., Urschel, C. W., Sonnenblick, E. H. and La Raia, P. J. (1976) J. Mol. Cell. Cardiol. **8**, 521-531

Suzuki, Y., Kamikawa, T., Kobayashi, A., Masumara, V. and Yamazaki, N. (1981) Jpn. Circ. J. **45**, 687-694

Takeo, S., Tananaka, K., Shimiza, K., Hirai, K., Miyake, K. and Minematsu, R. (1989) J. Pharm. Exp. Ther. **248**, 306-314

Tanaka, M., Gilbert, J. and Pappano, A. J. (1992) J. Mol. Cell. Cardiol. **24**, 711-720

Tani, M. and Neely, J. R. (1989) Circ. Res. **65**, 1045-1056

Tani, M. and Neely, J. R. (1990) J. Mol. Cell. Cardiol. **22**, 57-72

Tutwiler, G. F., Kirsch, T., Mohrbacher, R. J. and Ho, W. (1978) Metabolism. **27**, 1539-1556

van Bilsen, M., van der Vusse, G.J., Willemsen, P. H. M., Coumans, W. A., Roemen T. H. M. and Reneman, R. S. (1989) Circ. Res. **64**, 304-314

van Bilsen, M., van der Vusse, G. J., Willemsen, P. H. M., Coumans, W. A., Roemen, T. H. M. and Reneman. R. S. (1990) J. Mol. Cell. Cardiol. **22**, 155-163

van Bilsen, M., van der Vusse, G.J., Willemsen, P. H. M., Coumans, W. A., Roemen T. H. M. and Reneman, R. S. (1991) J. Mol. Cell. Cardiol. **23**, 1437-1447

van der Merwe, E., Harper, I. S., Owen, P., Lochner, A., Wynchank, S. and Opie, L.H. (1985) Mol. Physiol. **8**, 285-287

van der Vusse, G. J., Roemen, Th. H. M., Prinzen, F.W., Coumans, W. A. and Reneman R. S. (1982) Circ. Res. **50**, 538-546

van der Vusse, G. J., Roemen, T. H. M., Flameng, W. and Reneman, R. S. (1983) Biochim. Biophys. Acta. **752**, 361-370

van der Vusse, G. J., Prinzen, F. W., van Bilsen, M., Engels, W. and Reneman, R. S. (1987) in Lipid metabolism in the normoxic and ischaemic heart (Stam, H. and van der Vusse, G. J., eds.) pp. 157-167

Vaughan Williams, E. M. (1959) J. Physiol. **149**, 78-92

Vetter, N.J., Strange, R.C., Adams, W. and Oliver, M.F. (1974) Lancet. **1**, 284-289

Vik-Mo, H. and Mjos, O. D. (1981) Am. J. Cardiol. **48**, 361-365

Vik-Mo, H., Riemersma, R.A., Mjos, O.D. and Oliver, M.F. (1979) Scand. J. Clin. Lab. Invest. **39**, 559-568

Vik-Mo, H., Riemersma, R.A., Mjos, O.D. and Oliver, M.F. (1981) Adv. Physiol. Sci. **8**, 121-128

Vik-Mo, H., Mjos, O. D., Neely, J. R., Maroko, P. R. and Riberio, L. E. T. (1986) Am. Heart. J. **111**, 1048-1054

Vleugels, A., Vereecke, J. and Carmeliet, E. (1980) Circ. Res. **47**, 501-508

Weglicki, W. B., Owens, K., Ruth, R. C. and Sonnenblick, E. H. (1974) Cardiovasc. Res. **9**, 237-242

Weishaar, H. D. (1975) Med. Well. 26, 387

Weishaar, R., Sarma, J. S. M., Maryama, Y., Fishmer, R. and Bing, R. J. (1977) Cardiology. **62**, 2-20

Weiss, J. N. and Hiltbrand, B. (1985) J. Clin. Invest. **75**, 436-447

Weiss, J. N. and Lamp, S. T. (1987) Science. **238**, 67-69

Weiss, R. G, Chacko, V. P and Gerstenblith, G. (1989) J. Mol. Cell. Cardiol. **21**, 469-478

Weissler, A. M., Kruger, F. A., Baba, N., Scarpelli, D. G., Leighton, R. F. and Gallimore, J. C. (1968) J. Clin. Invest. **47**, 403-416

Whitehouse, S. and Randle, P. (1973) Biochem. J. **134**, 651-653

Wojtczak, L. (1974) FEBS. Lett. **44**, 25-30

Woldegiorgis, G., Shrago, E., Gipp, J. and Yatvin, M. (1981) J. Biol. Chem. **256**, 12297-12300

Woldegiorgis, G., Yousufzai, S. Y. K. and Shrago, E. (1982) J. Biol. Chem. **257**, 14783-14787

Wolf, H. P. O., Eistetter, K., and Ludwig, G. (1981) Diabetologia. **21**, 344

Wollenberger, A. and Krause, E. G. (1968) Am. J. Cardiol. **22**, 349-359

Wollenberger, A., Krause, E. G. and Shahab, L. (1967) in International Symposium on the coronary circulation and energetics of the myocardium (Marchetti, G. and Taccardi, B., eds.) pp. 200-219, S. Karger, Basle

Wood, J. M., Bush, B., Pitts, B. J. R. and Schwartz, A. (1977) Biochim. Biophys. Res. Comm. **74**, 677-684

Worgovitch, T. J., MacDonald, R. G., Hill, J. A., Feldman, R. L., Stacpoole, P.W. and Pepine, C. J. (1988) Am.J.Cardiol. **61**, 65-70

Worthington, M. G. and Opie, L. H. (1992) J. Cardiovasc. Pharmacol. **20**, 595-600

Yang, Z., Richard, V., von Segesser, L., Bauer, E., Stultz, P., Turina, M. and Luscher, T. F. (1990) Circulation. **82**, 188-195

Zimmerman, A. N. E. and Hulsmann, W. C. (1966) Nature. **211**, 646-647

Publications

Clarke, B., Spedding, M., Patmore, L. and McCormack, J. G. (1993) Protective effects of ranolazine in guinea-pig hearts during low-flow ischaemia and their association with increases in active pyruvate dehydrogenase. Br. J. Pharmacol. **109**, 748-750

GLASGOW
ERSITY
LIBRARY