The Regulation of Heart AMP-Activated Protein Kinase by Long-Chain Fatty Acids and Hormones.

by

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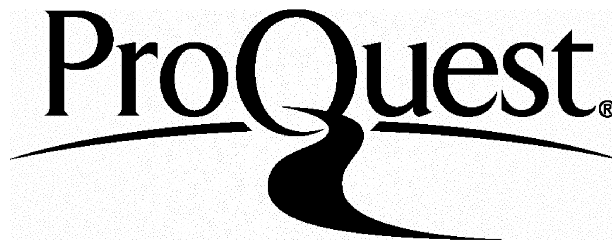

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Abstract.

Malonyl-CoA plays a role in the regulation of cardiac β-oxidation due to its ability to inhibit carnitine palmitoyltransferase 1. Acetyl-CoA carboxylase (ACC) is the enzyme responsible for the synthesis of malonyl-CoA, the concentration of which increases in hearts exposed to glucose and insulin and is decreased by physiological concentrations of fatty acids and by adrenaline.

ACC is phosphorylated and inhibited in response to the activation of the AMP-activated protein kinase (AMPK) cascade. Both α-1 and α-2 AMPK isoforms are phosphorylated and activated in hearts perfused with physiological concentrations of palmitate. This activation is not associated with alterations in the ratio of AMP to ATP. The activity of both AMPK isoforms is decreased after exposure to insulin, an effect overcome by 0.5mM palmitate. The activity of α-1 AMPK is stimulated in glucose perfused hearts by adrenaline, with rates similar to those measured in hearts perfused with 0.5mM palmitate. Adrenaline failed to increase α-1 AMPK activity above that due to fatty acids in palmitate perfused hearts. Adrenaline stimulation of palmitate perfused hearts resulted in a reversal of the effect of this fatty acid on α-2 AMPK activity. Adrenaline was without effect on the activity of this isoform in hearts perfused with glucose as the sole substrate.

ACC was highly phosphorylated at the AMPK site in tissue from hearts exposed to 0.5mM palmitate compared to those perfused with glucose alone. However despite this, no alteration in ACC activity could be measured in response to palmitate and hormones in this study except for a small decrease due to adrenaline in palmitate perfused hearts.

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Abbreviations.

ACC	Acetyl-CoA carboxylase
AICAR	5'-aminoimidazole-4-carboxamide riboside
AMPK	5' -AMP-activated protein kinase
AMPKK	5'-AMP-activated protein kinase kinase
CAT	Carnitine acetyltransferase
CoASH	Coenzyme A
CPT	Carnitine palmitoyltransferase
DTT	Dithiothreitol
F2,6BP	Fructose 2,6-bisphosphate
F-6-P	Fructose 6-phosphate
FAD	Flavin adenine dinucleotide (oxidised)
$FADH_2$	Flavin adenine dinucleotide (reduced)
FAS	Fatty acid synthase
G-6-P	Glucose 6-phosphate
HSL	Hormone sensitive lipase
LPL	Lipoprotein lipase
MCD	Malonyl-CoA decarboxylase
MEHA	3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline
NAD^+	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NEFA	Non-esterified fatty acid
PEG	Polyethylene Glycol
PDH	Pyruvate dehydrogenase complex
PFK	Phosphofructokinase
PKA	Cyclic-AMP-dependent protein kinase

PMSF	Phenylmethylsulfonyl fluoride
PP2C	Phosphoprotein phosphatase 2C
PVDF	Polyvinylidene fluoride
SAMS	Synthetic peptide based on the AMPK site of rat ACC
TAG	Triacylglycerol
ZMP	5'-aminoimidazole-4-carboxamide riboside monophosphate

For my dad, James Stewart Clark.

Chapter One

Introduction.

1.1. General Introduction.

The myocardium is able to generate energy in the form of adenosine triphosphate (ATP) from a variety of metabolic fuels in order to sustain contractile function. These include fatty acids and carbohydrates from the diet as well as compounds produced by metabolism such as ketones, pyruvate and lactate. Despite its omnivorous nature, long chain fatty acids are used preferentially as a myocardial fuel source and provide 60-70% of the heart's energy needs.

Tightly regulated mechanisms for fuel selection operate within the myocardium. The relative contribution of each substrate to energy production varies depending on several parameters, including substrate availability, workload and hormonal stimulation. The rate of utilization of each substrate is also regulated. Mechanisms include the control of exogenous substrate extraction from the circulation, regulation of the turnover of endogenous fuel stores, and the control of enzymes in catabolic pathways.

The following sections focus upon the control of circulating fatty acid and glucose uptake and the mechanisms by which energy from endogenously stored substrate can be harnessed. The regulation of the major energy generating pathways is also discussed, with the effects of alternative substrate provision, hormones and disease states on cardiac fuel selection also outlined in detail.

1.2. Transport of Exogenous Fuels.

1.2.1. Glucose.

The rate of glucose utilization in the heart is primarily linked to the rate of glucose uptake into the cell. This process is mediated by facilitated diffusion through specific glucose transporters, and the rate is controlled by the concentration gradient, the number of transporters and their affinity for substrate (King L.M. and Opie L.H, 1998). The metabolic requirements of the cell also play a role in determining the rate of glucose uptake. Such

requirements include dietary state, hormones such as insulin, glucagon and catecholamines, and the availability of alternative substrates.

Once inside the cell glucose is phosphorylated by the action of hexokinase (Section 1.4.1.1.) to produce glucose-6-phosphate. This reaction is irreversible in the heart under physiological conditions, as unlike liver, glucose-6-phosphatase is not expressed in cardiomyocytes (Neely JR and Morgan HE, 1974). Glucose transport is rate limiting in unstimulated perfused rat hearts (Morgan HE *et al.*, 1961b); increasing extracellular glucose results in an increase in glucose-6-phosphate without an increase in intracellular free glucose. The hexokinase reaction, however, becomes limiting when glucose transport is stimulated, for example by insulin (Morgan HE *et al.*, 1961b), and there is an accumulation of free glucose inside the cell (Manchester J *et al.*, 1994).

Left ventricular pressure development in both Langendorff and working rat hearts results in a stimulation of glucose transport as well as increasing glucose phosphorylation (Neely JR *et al.*, 1967). The increase in glucose transport caused by pressure development in hearts perfused with glucose as the sole substrate is reversed by the addition of physiological concentrations of palmitate to the perfusion media (Neely JR *et al.*, 1969). This inhibitory effect of fatty acids is due to decreased membrane transport rather than phosphorylation, as no accumulation of free glucose is observed under these conditions.

1.2.1.1. Glucose Transporters.

There are two mechanisms for facilitated glucose uptake in animal cells. Na^+ - dependent glucose transporters, driven by the Na^+ gradient are not found in the heart. The specific glucose transporters in the heart are members of the GLUT family of transporters. Up to six isoforms of the GLUT transporter are found, which are distributed in accordance with the needs of individual tissue types (Pessin JE and Bell GI, 1992).

The major glucose transporter expressed on the cardiomyocyte is the GLUT4 isoform. The GLUT1 transporter which is present in most tissues is also found in cardiac myocytes, but is only one fifth as abundant as GLUT4 (Kraegen EW *et al.*, 1993). GLUT1 has a higher affinity for glucose and is

likely to be responsible for efficient transport at low circulating glucose concentrations. Both transporters are found distributed between the plasma membrane and stored in cytosolic vesicles, and translocation to the membrane is accelerated by insulin, with GLUT4 being the most insulin responsive (Pessin JE and Bell GI, 1992).

The rate of glucose transport is mainly controlled by the number of transporters present at the cell surface. In the short-term this number is determined by the rate of recruitment of transporters from intracellular stores, whereas the rate of protein synthesis is important for long–term control (King L.M. and Opie L.H, 1998).

The number of glucose transporters present at the cell surface is increased by hormones, lack of oxygen and increasing the energy demands of the heart and is decreased by alternative substrates including fatty acids. Products of intermediary metabolism have been shown to decrease glucose transport in isolated cardiac myocytes, with pyruvate, lactate and propionate being most effective (Fischer Y *et al.*, 1997). The same authors reported that pyruvate caused a decrease in the number of GLUT4 transporters on the myocyte surface, and also reduced numbers of GLUT1 transporters in phenylephrine stimulated cells.

1.2.2. Fatty acids.

Cardiac myocytes have a low capacity for *de novo* fatty acid synthesis and so rely on a supply from the circulation. These fatty acids may be oxidized to provide energy, or esterified to form an endogenous TAG store.

Fatty acids are present in the blood as either unesterified molecules, or incorporated into phospholipids, cholesteryl esters or as acylglycerols. The solubility of free fatty acids in plasma is increased by the formation of fatty acid –albumin complexes. Over 90% of plasma fatty acids are present as esters, which associate with proteins other than albumin to increase their solubility (Fredrickson DS and Gordon RS, 1958). Blood borne TAG is an important source of myocardial fatty acids. These hydrophobic TAG molecules form the core of lipoprotein particles, such as chylomicrons and Very Low Density Lipoproteins (VLDL) which are surrounded by a hydrophilic layer composed of phospholipids, cholesterol and apoproteins.

Transport of fatty acids to the myocardium requires that they first cross the endothelial cell barrier surrounding the myocardium. Due to its large size albumin is not able to diffuse between endothelial cells, and transport of fatty acids through the endothelium in complex with albumin is unlikely (Van Der Vusse GJ *et al.*, 1992). The release of fatty acids from albumin is believed to involve the binding of albumin to the luminal surface of the endothelial cell followed by direct transfer of the fatty acids (Bassingthwaighte JB *et al.*, 1989).

Although endothelial cells are capable of internalising lipoprotein particles, the majority of fatty acids transported as TAG are obtained by its hydrolysis at the endothelial cell surface by the action of lipoprotein lipase (Cryer A, 1989).

The mechanism by which fatty acids are transported to the myocyte across the endothelium is not completely understood. It has been suggested that the fatty acids are transported by diffusion, down a concentration gradient, through a continuum of cell membranes without entering the endothelial cell cytoplasm (Scow RO and Blanchette-Mackie EJ, 1992). This idea has been criticised on the grounds that the process is too slow to be of physiological consequence, (Bassingthwaighte JB *et al.*, 1989) and that in heart there are no membrane continuities between endothelial cells and myocytes (Cryer A, 1989), and therefore transfer must be across the endothelium, mediated by fatty acid binding proteins (FABP).

Transport through the interstitial space from the endothelium to the myocyte is most likely mediated by albumin. The rate of fatty acid uptake by the myocyte is determined mainly by the rate of cellular metabolic reactions, and can be inhibited by competing substrates such as pyruvate and acetoacetate (Olson RE, 1962). This process may also be mediated by interactions between albumin: fatty acid complexes and the cardiac sarcolemma (Van Der Vusse GJ *et al.*, 1992), although a specific albumin binding site has not been identified. It has also been proposed that transsarcolemmal fatty acid uptake is mediated by a membrane bound FABP. Such a protein has been identified in a variety of cells, including cardiomyocytes. Antibodies raised against this $FABP_{PM}$ inhibit the influx of oleate by 40% (Stremmel W, 1998).

1.3. Endogenous Fuels.

The heart contains fuel stores for utilization during periods of increased cardiac work or hormonal stimulation when energy needs cannot be immediately met by exogenous substrates. Glucose is found stored in the myocardium in the form of glycogen. The heart also contains a small but significant store of fatty acids in the form of TAG. The following sections describe the mechanism by which turnover of these stores is regulated.

1.3.1. Glycogenolysis.

Glycogen is a readily mobilized storage form of glucose, consisting of between 10,000 and 30,000 glucose molecules bound by α-1,4 glycosidic linkages. Branches are created at about every tenth residue by α-1,6 bonds (King L.M. and Opie L.H, 1998).

Glycogen breakdown occurs mainly after adrenergic stimulation, during oxygen deprivation and on glucagon treatment (Cornblath M *et al.*, 1963). Palmitate has been shown to inhibit glycogenolysis in the heart, an effect which is overcome by catecholamines (Crass MF *et al.*, 1975). During increases in workload the heart has been shown to preferentially oxidize glycogen (Goodwin GW *et al.*, 1998). Glycogenolysis involves the cleavage of an α-1,4 bond from the glycogen macromolecule. The released glucose molecule is then phosphorylated to form glucose-1-phosphate. This reaction is catalysed by glycogen phosphorylase without the utilization of ATP, and prevents glucose diffusion from the cell (King L.M. and Opie L.H, 1998). Glucose -1-phosphate is then converted by phosphoglucomutase to glucose-6-phosphate, which is then able to enter the glycolytic pathway (Section 1.4.1).

Phosphorylase is unable to cleave α-1,6 linkages and stops cleaving the macromolecule three residues from a branch point. In order for glycogenolysis to continue, other enzymes are needed. A transferase transfers three residues from one branch to another, whilst a second, α-1,6 glucosidase (or debranching enzyme), hydrolyses the α-1,6 bond at the branch point. In this way the two enzymes convert the branched structure into a linear one, allowing further cleavage by phosphorylase.

1.3.1.1. Glycogen Phosphorylase.

Glycogen phosphorylase is regulated by allosteric effectors, which signal the cellular energy status, and by reversible phosphorylation in response to hormones. The enzyme exists in two forms, known as phosphorylase a and phosphorylase b (Neely JR and Morgan HE, 1974). The a form is the active phosphorylated form, which does not require AMP for activation and is inhibited by glucose. Phosphorylase b is dephosphorylated and is dependent on AMP for activity. The b form is also inhibited by ATP and glucose-6-phosphate. Activity of phosphorylase is regulated by phosphorylation /dephosphorylation reactions, catalysed by phosphorylase kinase and phosphorylase phosphatase (see Figure 1.1.).

The increase in glycogen breakdown due to catecholamines is caused by activation of phosphorylase kinase by increases in intracellular [Ca^{++}], as well as by cyclic AMP-dependent protein kinase (PKA), and phosphorylase b to a conversion. The increase in glycogenolysis seen during anoxia is not prevented by treatment with β-adrenergic antagonists (Dobson JG and Mayer SE, 1973). This effect is likely to be caused by rises in the level of AMP which allosterically activate phosphorylase b, as well as increases in orthophosphate, the phosphorylase substrate (Morgan HE and Parmeggiani A, 1964).

1.3.2. Triacylglycerol.

In the myocardium the endogenous TAG store contains approximately 50μmol of esterified fatty acids/ g dry weight which is able to support the energy needs of the heart for almost 45 minutes at normal heart rate (Olson RE and Hoeschen RJ, 1967). The TAG store is the only lipid pool which contributes significant amounts of fatty acids for oxidative metabolism, with the contributions from phospholipids, cholesterol esters and mono- and diacylglycerols amounting to only 5-10% (Saddik M and Lopaschuk GD, 1991). In the same study fatty acids derived from TAG were shown to meet 50% of the energy requirements of the working rat heart when perfusions were carried out in the absence of exogenous fatty acids.

Triacylglycerols are present throughout the heart tissue with a large proportion contained within cardiac adipocytes. In addition TAG is stored in

Figure 1.1. Control of Glycogenolysis.

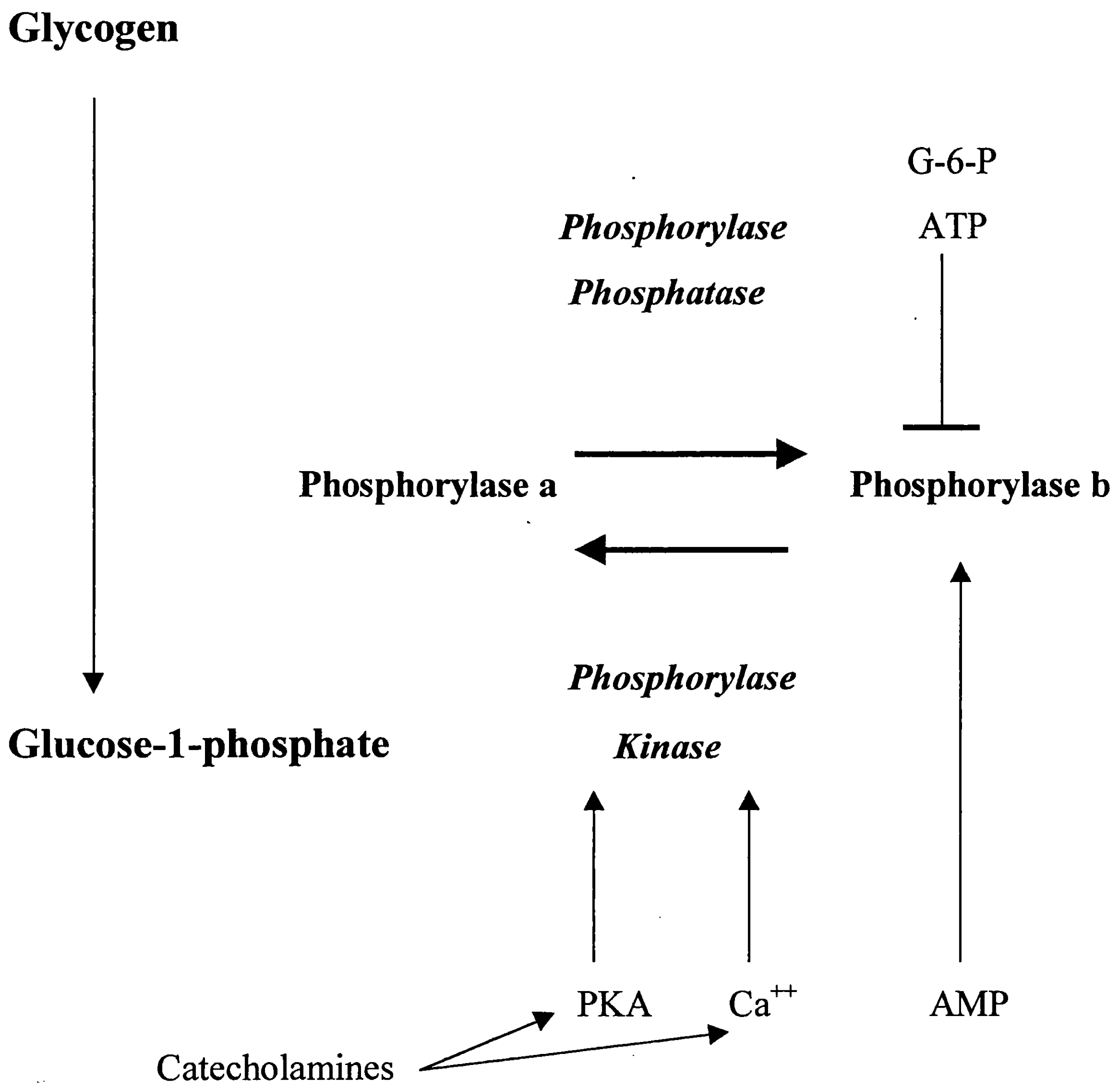


Figure 1. Glycogen phosphorylase is the enzyme responsible for cleaving glycogen to produce glucose-1-phosphate. Phosphorylase a is the active, phosphorylated form of the enzyme. Glycogen breakdown is inhibited by the action of phosphorylase phosphatase which converts phosphorylase a to the inactive, dephosphorylated b form. Catecholamines increase glycogen breakdown by activation of phosphorylase kinase and therefore phosphorylase b to a conversion. Phosphorylase b can be activated when AMP levels increase, signalling low fuel availability, and is inhibited by ATP and glucose-6-phosphate. Adapted from King and Opie, 1998.

the myocyte, with small amounts present in endothelial cells. These endogenous TAGs are found as free floating cytoplasmic droplets in close proximity to mitochondria. Intracellular TAG is also found in association with lysosomes (Stam HCG *et al.*, 1987). TAG found in the sarcoplasmic reticulum is likely to be newly synthesised and may not be available for hydrolysis (Christiansen K, 1975).

Whether myocardial TAG exists purely as an energy store or as a metabolic intermediate is not clear. Substantial TAG turnover occurs in hearts perfused with high (1.2mM) fatty acid (Saddik M and Lopaschuk GD, 1991), suggesting that the endogenous TAG pool is dynamic with continuous lipolysis and resynthesis occurring simultaneously. In isolated cardiac myocytes with TAG stores prelabelled with [^{3}H]-palmitate, loss of the [^{3}H] label from TAG is greater than rates of lipolysis calculated on the basis of glycerol release (Swanton EMS and Saggerson ED, 1997). This can be explained by a cycle of partial hydrolysis to mono- or diacylglycerol followed by re-esterification to TAG. Addition of adrenaline to the myocyte incubation medium abolished this cycling, when loss of [^{3}H] from TAG and glycerol release became equal.

The rate of lipolysis in the heart is linked to the immediate energy requirements of the organ and is determined by exogenous substrate availability, hormonal stimulation and cardiac work.

Increases in ventricular pressure development in working rat hearts stimulates rates of TAG mobilisation and endogenous lipid oxidation (Crass MF *et al.*, 1971). Increased rates of glycerol release in the isolated perfused heart are seen when contraction is stimulated by glucagon, isoproterenol and ouabain (Jesmok G.J. *et al.*, 1976). The stimulatory effect of isoproterenol could be overcome by the β-adrenergic antagonist propranolol. The increase in lipolysis associated with glucagon and isoproterenol was associated with increases in the PKA activity
ratio, indicating the effect of these hormones is mediated by cAMP. No change in the PKA activity ratio was observed with ouabain, suggesting that control of cardiac lipolysis does not depend solely on cAMP.

Extracellular [Ca^{++}] is important for glucagon and isoproterenol stimulated lipolysis (Hron W.T. *et al.*, 1977), with no increases in glycerol release

observed until the concentration reached a threshold level of 1.25mM, despite increases in the PKA activity ratio.

Isoproterenol stimulated lipolysis can be inhibited in perfused rat hearts by ketone bodies (Hron W.T. *et al.*, 1978). Again this effect was not mediated by preventing cAMP accumulation. Other studies with isolated cardiac myocytes have shown no inhibition of glycerol release by ketones (Larsen T.S. and Severson D.L., 1990), indeed a stimulation of lipolysis was observed in control (TAG depleted) myocytes in the basal and isoproterenol stimulated state. Ketones produced no change in lipolysis in TAG loaded myocytes in the same study.

Isolated cardiac myocytes show reduced levels of basal and catecholamine stimulated lipolysis in the presence of exogenous fatty acids (Larsen T.S. and Severson D.L., 1990; Swanton EMS and Saggerson ED, 1997). In isolated working rat hearts with TAG stores prelabelled with [^{14}C]-palmitate, production of $^{14}CO_2$ decreased as the exogenous fatty acid supply was increased (Crass MF, 1972; Saddik M and Lopaschuk GD, 1991). Perfusion of hearts with exogenous fatty acids results in an increase in tissue levels of free fatty acids and fatty acyl-CoA (Oram JF *et al.*, 1973) and it has been proposed that tissue levels of these lipolytic products may play a role in regulating lipolysis in response to the availability of exogenous fatty acid supply. Severson and Hurley, 1982 have shown that free fatty acids and fatty acyl-CoA are able to inhibit neutral TAG lipase activity in a partially purified enzyme preparation from rat heart. However, the concentrations of acyl-CoAs utilised in this study were high (25-100μM) as 10μM is believed to be the upper limit for heart acyl-CoA concentration (Van Der Vusse GJ *et al.*, 1982).

Insulin has no effect on lipolysis in the perfused rat heart, a situation which differs from adipose tissue where the hormone shows antilipolytic activity (Christian DR *et al.*, 1969).

1.3.2.1. Myocardial TAG Lipases.

The availability of TAG for enzymatic hydrolysis is likely to depend on its intracellular location and the proximity of TAG lipases. The rate limiting step in TAG breakdown is hydrolysis of the first fatty acid to produce diacylglycerol (DAG), as the activity of DAG and monoacylglycerol (MAG) lipases exceed

the TAG lipase activity in cardiac tissue (Severson D.L., 1979; Stam HCG *et al.*, 1986). The identity of this key TAG lipase has been a matter of major debate.

The heart contains a number of TAG lipases with both acid and neutral pH optima. Acid lipases are likely to be located in cardiac lysosomes and have been proposed to be the candidate lipases for the hydrolysis of endogenous TAG. TAG is found in lysosomes, and levels become depleted during *in vitro* heart perfusion, although this decrease represents only 10% of the total TAG loss. This decrease in TAG content is also seen in hearts with depleted neutral TAG lipase activity (Stam HCG *et al.*, 1986). The role of lysosomal TAG lipases in mediating endogenous TAG hydrolysis has been challenged on the grounds that cardiac lipolysis was unaffected by the lysosomotrophic agent methylamine in both basal and isoproterenol stimulated incubations (Kryski A. *et al.*, 1987).

Two other intracellular TAG lipases which have been reported to play a role in endogenous TAG hydrolysis are lipoprotein lipase and hormone-sensitive lipase.

1.3.2.2. Lipoprotein Lipase.

Lipoprotein Lipase (LPL) is located in two distinct fractions in heart tissue. The largest pool is intracellular and has been referred to as the 'non-functional' enzyme (Borensztajn J *et al.*, 1975). A smaller pool is located at the endothelial cell surface and has been referred to as a 'clearing factor' lipase which hydrolyses circulating lipoprotein TAG to fatty acids and glycerol for transport into the myocyte. The intracellular LPL is a storage pool of this extracellular lipase.

Endothelial LPL is synthesised within the myocyte with evidence existing showing that the gene encoding the lipase is found exclusively within these cells (Camps L *et al.*, 1990). The enzyme is processed via the secretory pathway and transported to the endothelial surface. The amount of LPL at the endothelial cell surface depends upon rates of synthesis of the enzyme, rates of transport versus rates of degradation within lysosomes, and the amount of LPL released into the interstitial space for transfer to the luminal surface of the endothelium. Fatty acids generated by lipoprotein hydrolysis

cause release of LPL from cultured endothelial cells, suggesting that the products of the lipase reaction are able to regulate amounts of enzyme bound to the endothelial cell (Saxena U *et al.*, 1989). Once released from the heart the enzyme is degraded by the liver and not internalised by the heart tissue (Cryer A, 1989).

The rate of hydrolysis of circulating TAG is largely dependent on TAG concentration in the circulation and the amount of LPL at the endothelial luminal surface. Full activity of LPL also depends on the presence of apoprotein CII which is found on the surface of lipoproteins. Accumulation of free fatty acids and monoacylglycerols inhibit the hydrolytic activity of LPL (Olive-Crona T *et al.*, 1987).

Short-term regulation of LPL activity is mainly due to alterations in rates of secretion and loss of enzyme from the endothelial cell surface. The quantity and activity of LPL bound to the endothelium varies depending upon nutritional state and have been shown to increase during fasting (Borensztajn J *et al.*, 1970) and fat feeding (Jansen H *et al.*, 1975). Exogenous heparin is able to cause the release of LPL from the endothelial cell. Adrenaline and glucagon increase the activity of heparin-releasable LPL in perfused rat hearts, with a corresponding decrease in the non-functional (intracellular) LPL activity (Stam HCG and Hülsman WC, 1984). These observations indicate a hormone stimulated increase in LPL transport from the intracellular compartment of the myocyte to the site of enzyme action. Propranolol has been found to block the increase in LPL transport caused by adrenaline, indicating that transport is mediated by increases in cAMP (Stam HCG and Hülsman WC, 1984).

Long-term regulation of LPL activity is likely to occur by alterations in rates of enzyme synthesis and degradation within the myocyte. Rats treated with corticosteroids, adrenocorticotrophic hormone (ACTH) and thyroxine showed a long-term stimulation of total LPL activity. Diabetes has also been shown to increase LPL activity in the heart (Stam HCG *et al.*, 1984). The increase in LPL activity which occurs during fat feeding is likely to be due to glucocorticoids (Pedersen ME *et al.*, 1981). Unlike skeletal muscle where it causes a decrease in LPL synthesis, in the heart insulin does not affect LPL activity (Stam HCG *et al.*, 1984).

It now seems unlikely that LPL is the enzyme responsible for intracellular TAG mobilisation. The intracellular 'non-functional' LPL activity resides in secretory vesicles where it is not in direct contact with cytosolic lipid droplets. Secondly, the enzyme dependence on serum and apo CII give support to it having a solely extracellular function.

1.3.2.3. Hormone-Sensitive Lipase.

The presence of a neutral TAG lipase in heart, distinct from LPL was first shown in 1985 (Ramirez I. *et al.*, 1985), and shown to be activated by PKA (Goldberg D.I. and Khoo J.C., 1985). This lipase is now thought to be hormone-sensitive lipase (HSL) and the enzyme responsible for hormonally stimulated myocardial lipolysis.

HSL was previously considered to be an adipose tissue specific TAG lipase. The enzyme was purified in 1981 by Fredrikson *et al,* and found to have much higher catalytic activity towards DAG compared to TAG. This observation is in keeping with measurements of lipase activity in the heart, where hydrolysis of TAG is the rate limiting reaction in TAG breakdown. HSL also has cholesterol esterase activity equivalent to its TAG lipase activity. HSL has since been shown to be present in tissues other than adipocytes including heart and skeletal muscle (Holm C *et al.*, 1987; Small CA *et al.*, 1989).

HSL activity can be regulated by phosphorylation /dephosphorylation reactions. Two sites have been identified which are phosphorylated both *in vitro* and *in vivo.* Residue 563 has been identified in rat HSL as the position which is phosphorylated by PKA in response to lipolytic stimulation (Garton A.J. *et al.*, 1988). A second 'basal' phosphorylation site is located at residue 565 in the rat sequence (Strålfors P *et al.*, 1984). A number of protein kinases act at this site, including the AMP-activated protein kinase (AMPK) (Garton A.J. *et al.*, 1989). Phosphorylation of these sites has been shown to be mutually exclusive *in vitro,* with phosphorylation at one site blocking subsequent phosphorylation at the second (Garton A.J. and Yeaman SJ, 1990). Two additional sites, ser-659 and ser-660, have been shown to be phosphorylated in isoproterenol-stimulated adipocytes (Anthonsen MW *et al.*, 1998), with mutation of these sites but not of ser-563 abolishing HSL activation by PKA. Activity of HSL can also be regulated by protein phosphatases. HSL is a substrate *in vitro*, of protein phosphatase 1, protein

phosphatase 2A and 2C. The level of phosphatase activity at each site is unclear. One study has shown that each phosphatase has greater activity toward the basal site compared to the regulatory site (Olsson H and Belfrage P, 1987). A later study, however has shown high phosphatase activity toward both the basal and regulatory sites (Wood SL *et al.*, 1993).

Rates of lipolysis in adipocytes stimulated by noradrenaline increase by up to 100 fold, whereas *in vitro* dephosphorylated HSL activity increases only 2-3 fold when fully activated by PKA (Fredrikson G *et al.*, 1981), therefore changes in HSL activity are insufficient to account for the increase in lipolysis. Recently evidence has suggested that phosphorylation by PKA also promotes a translocation of HSL to the cytosolic lipid, targeting the enzyme to its substrate. Egan *et al,* 1992 have shown using anti-HSL antibodies in 3T3-L1 adipocytes that HSL is distributed throughout the cytoplasm under basal conditions and moves to the lipid droplet upon lipolytic stimulation.

In adipocytes a role has also been proposed for the phosphoprotein perilipin in regulating association of HSL with its substrate. Perilipin is located at the site of HSL action, on the surface of the lipid droplet (Greenberg AS *et al.*, 1991), and is itself a substrate for phosphorylation in response to lipolytic hormones. Perilipin has been proposed to function as a docking protein which when phosphorylated serves to anchor HSL to its substrate (Egan JJ *et al.*, 1992). However, more recent work has shown that although stimulation of lipolysis in young rats was associated with HSL translocation, in older rats where less lipolytic stimulation was observed, no translocation of HSL was observed despite perilipin being highly phosphorylated (Clifford GM *et al.*, 2000). Translocation of perilipin away from the lipid droplet does not seem to be important for increasing HSL access to its substrate. In the above study perilipin translocation was observed in adipocytes from older, less adrenergically responsive rats, but not in tissue from young rats in which catecholamine induced lipolysis was greater.

1.4. Fuel Catabolism.

1.4.1. Glycolysis.

Glycolysis is the sequence of reactions catalysing the conversion of one molecule of glucose into two molecules of pyruvate with the net production of two molecules of ATP.

Glucose utilization is regulated at several points in the glycolytic pathway. The significance of this regulation is controversial with uncertainty whether control is at a few defined reactions, or distributed along the length of the pathway. It has been proposed that glycolytic inhibition during ischaemia is controlled at the level of glyceraldehyde-3-phosphate dehydrogenase as accumulation of intermediates prior to this step occurred (Rovetto MJ *et al.*, 1975). More recently it has been shown that control of the glycolytic flux is not exerted by a single enzyme but occurs at multiple points in the pathway (Kashiwaya Y *et al.*, 1994). These authors suggest that the control of glycolysis lies mainly at the level of glucose transport and phosphorylation, with only 25% of control mediated by reactions below phosphoglucoisomerase, and that the proportion of control at each step varies depending on alternative substrate availability and hormonal stimulation. However the three non-equilibrium glycolytic enzymes, hexokinase, phosphofructokinase 1 and pyruvate kinase are the main candidates for controlling flux through the pathway (Figure 1.2.).

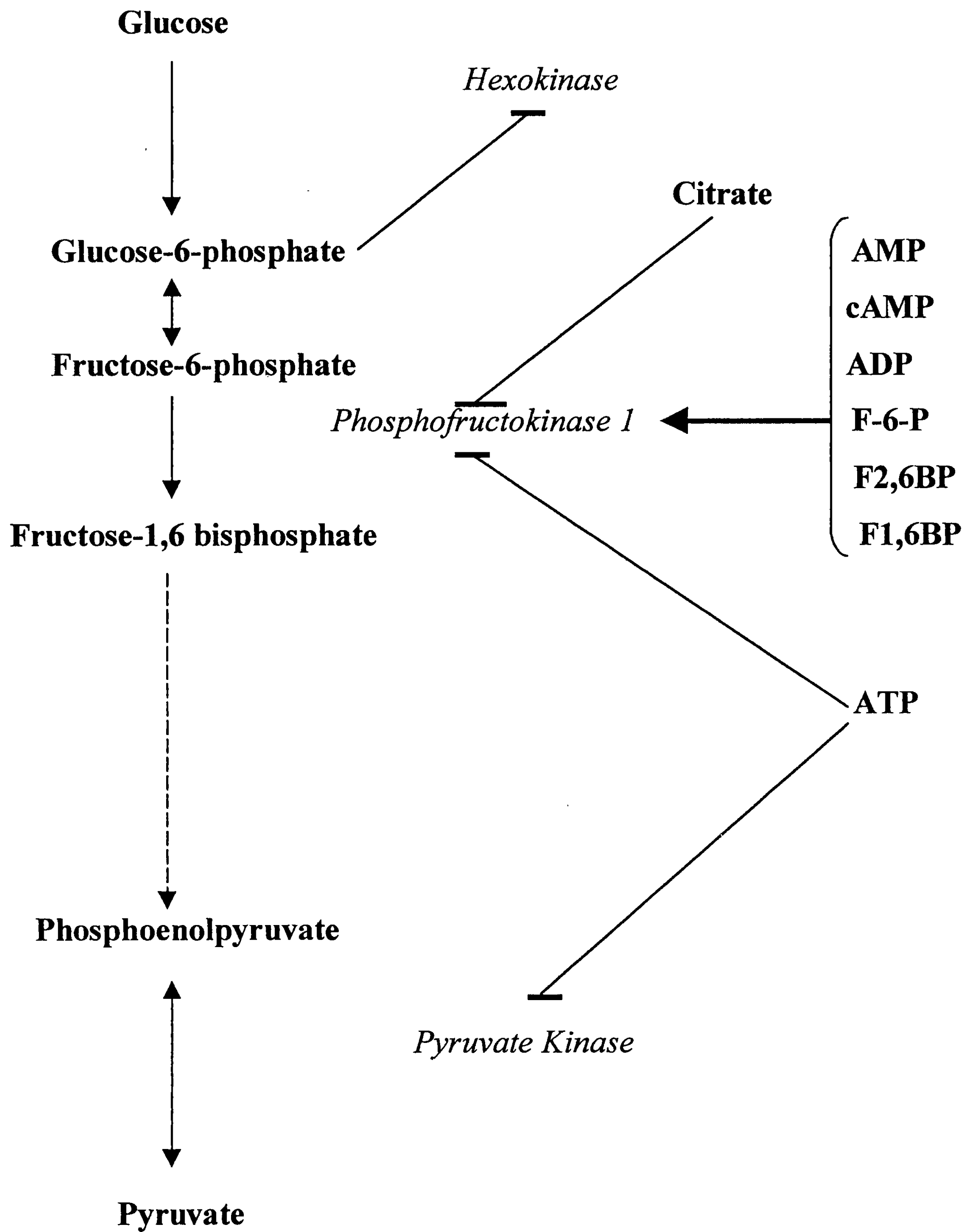
1.4.1.1. Hexokinase.

Hexokinase (HK) is the enzyme responsible for the phosphorylation of cytosolic glucose to form glucose-6-phosphate (G-6-P), which is not able to diffuse from the cell.

Hexokinase is inhibited *in vitro* by its product G-6-P, although whether this occurs *in vivo* is not clear. Increased rates of glucose transport and phosphorylation are seen in the heart after glucose loading, insulin treatment and oxygen deprivation, despite increases in intracellular G-6-P (Depre C *et al.*, 1993).

HK can be distributed between the cytosol and the mitochondrial membrane where it is attached by its N-terminus to porin (Arora KK *et al.*, 1992). In

Figure 1.2. Control Of The Glycolytic Pathway.



normal cells very little HK is bound to the mitochondria, whilst in tumour cells which have increased fuel requirements binding to the mitochondria is increased. HK activity is increased by this binding, the effect due to increased access to ATP via the porin and loss of G-6-P inhibition (Arora KK and Pedersen DL, 1988).

1.4.1.2. Phosphofructokinase 1.

Phosphofructokinase 1 (PFK1) catalyses the first irreversible step of glycolysis, the phosphorylation of fructose-6-phosphate (F-6-P) to form fructose 1,6-bisphosphate (F1,6-BP). PFK is a major control point in the glycolytic pathway able to direct glucose toward either glycogen synthesis or pyruvate production.

PFK is controlled by a number of allosteric effectors. ATP (the enzyme substrate) has a negative effect, which can be relieved by an excess of F-6-P, the second substrate (Passoneau JV and Lowry OH, 1962). As it is sensitive to the ATP/AMP ratio, PFK1 is able to link glycolytic rates to the cellular energy state.

Other important allosteric regulators include protons (Ui M, 1966) and citrate (Garland P.B. *et al.*, 1963) which exert inhibitory effects. Citrate enhances inhibition by ATP, whilst proton inhibition of PFK1 may serve to prevent excessive lactate and H^+ accumulation. PFK1 activity is increased by AMP and fructose 2,6-bisphosphate (F2,6-BP). F2,6BP is formed from F-6-P and ATP by phosphofructokinase 2 (PFK2), in a reaction stimulated by F-6-P (Hue L *et al.*, 1995). When physiological concentrations of substrates and effectors are present PFK1 is inactive without the presence of F2,6-BP (Narabayashi H *et al.*, 1985), and changes in rates of glycolysis correlate with changes in F2,6BP concentration (Lawson JWR and Uyeda K, 1987).

PFK1 has been reported to be a substrate for protein kinases. PFK1 in rat heart is activated in response to epinephrine by increases in the concentration of the positive effectors F-6-P, F2,6BP and AMP as well as by covalent modification (Narabayashi H *et al.*, 1985). In this study covalent modification caused a decreased sensitivity to ATP and citrate inhibition and a high sensitivity to activation by F2,6BP. These results are in contrast with the effects of adrenergic agents in skeletal muscle. Phosphorylated muscle

PFK1 is more sensitive to inhibition by citrate and ATP and less sensitive to AMP activation than the dephosphorylated enzyme (Kitajima S *et al.*, 1983). PFK1 is also a substrate of protein kinase C (PKC), and phosphorylation causes activation and increases its affinity for F-6-P. This phosphorylation may not be physiologically relevant however, as it is prevented *in vitro* by F2,6BP (Hue L and Rider MH, 1987). Whether regulation by phosphorylation/ dephosphorylation reactions is important for PFK1 activity *in vivo* is still disputed.

1.4.1.2.1. Control of F2,6BP Concentration.

F2,6BP is a signalling molecule whose synthesis and degradation is controlled by the bifunctional enzyme 6-phosphofructo-2-kinase (PFK2)/ fructose-2,6-bisphosphatase (FBPase2). Tissue specific isoforms of this enzyme exist, in the heart two isoforms of 54 and 58kDa are found (Rider MH *et al.*, 1992). The activity of this enzyme is dependent upon its phosphorylation state as well as the cellular concentration of substrates and regulatory factors. The heart enzyme has far greater PFK2 than FBPase2 activity, and unlike isozymes in other tissues heart PFK2 is not sensitive to inhibition by glycerol-3-phosphate. An inverse correlation exists between tissue levels of F2,6BP and citrate levels in heart tissue and PFK2, like PFK1, is extremely sensitive to inhibition by citrate (Depre C *et al.*, 1998). FBPase2 is inhibited by F-6-P, and this can be relieved by glycerol-3-phosphate (Hue L and Rider MH, 1987). In this way increases in tissue levels of F-6-P result in increased F2,6BP, activation of PFK1 and a stimulation of glycolysis. Increasing the cellular citrate concentration results in inhibition of glycolysis, by both allosteric inhibition of PFK1 and by decreasing tissue levels of F2,6BP by inhibition of PFK2.

Hearts perfused with insulin or increasing glucose concentration show a stimulation of glycolysis that is associated with activation of PFK2 and increases in the tissue concentrations of both F-6-P and F2,6BP (Depre C *et al.*, 1993). Increasing the workload of perfused hearts also resulted in high glycolytic rates and a decrease in the Km of PFK2 for F-6-P. The inhibition of glycolysis seen when hearts are perfused with ketones correlates with decreased levels of F2,6BP (Hue L *et al.*, 1988), the same authors also measured a decrease in glycolysis in rat hepatocytes incubated with fatty

acids. These results were proposed to be a result of an increased citrate concentration causing inhibition of PFK2.

PKA phosphorylation of the liver enzyme results in an inactivation of PFK2 and a stimulation of FBPase2 activity. Different phosphorylation sites exist in the heart isoform and phosphorylation by PKA causes an activation of PFK2 with no effect on FBPase2 (Rider MH and Hue L, 1986). Heart, but not liver PFK2 is a substrate of PKC although phosphorylation produced no change in kinase or phosphatase activity (Rider MH and Hue L, 1986).

1.4.1.3. Pyruvate Kinase.

The final step in the glycolytic pathway, the formation of pyruvate and ATP from phosphoenolpyruvate and ADP is catalysed by pyruvate kinase (PK). The muscle isoform of PK is inhibited by ATP by its competitive binding at the ADP site and results in an accumulation of metabolites produced by the preceding enzymes of the pathway. Unlike the liver isoform, muscle PK is not inhibited by F1,6BP.

1.4.1.4. Pyruvate.

Under aerobic conditions the major fate of pyruvate is transport into the mitochondria where it can be converted into acetyl-CoA by the action of pyruvate dehydrogenase (PDH). When oxygen is lacking NADH accumulation causes inhibition of the citric acid cycle and pyruvate is converted to lactate by lactate dehydrogenase in a reaction which serves to regenerate cytosolic NAD^+ for the continuation of glycolysis. Lactate is then exported into the circulation via a monocarboxylate/proton transporter. Pyruvate also plays an important role in replenishing citric acid cycle intermediates. It can be converted to oxaloacetate by the action of pyruvate carboxylase, or transaminated to alanine with the concomitant production of α-ketoglutarate.

1.4.1.4.1. Pyruvate Dehydrogenase.

The regulation of PDH activity is an important mechanism in the overall control of glucose metabolism. The enzyme converts pyruvate to acetyl CoA with the reduction of NAD^+ (Pyruvate + CoASH + $NAD^+ \rightarrow$ Acetyl-CoA + CO_2 + NADH + H^+). PDH is a multienzyme complex located in the mitochondria

and in the heart its activity is controlled by the availability of alternative substrates, workload and hormones. PDH is regulated by phosphorylation/dephosphorylation reactions catalysed by PDH kinase and PDH phosphate phosphatase, with the dephosphorylated form being active. Glucose oxidation is decreased during starvation and diabetes, and in response to alternative substrates such as fatty acids and ketones, and correlates with the amount of PDH in the active form.

PDH kinase activity is stimulated by acetyl-CoA and NADH (Kerbey AL *et al.*, 1976) and inhibited by pyruvate and the pyruvate analogue dichloroacetate (McVeigh JJ and Lopaschuk GD, 1990). The stimulatory effect of pyruvate on PDH activity can be overcome by fatty acids and is impaired in diabetes. Fatty acids and ketones decrease the amount of PDH in the active form (Wieland O *et al.*, 1971). That oxidation of fatty acids is necessary for this conversion has been shown by the use of inhibitors of this process (Caterson ID *et al.*, 1982). Fatty acid oxidation results in an increase in the mitochondrial NADH/NAD^+ and acetyl-CoA/CoA ratios. These increases are able to cause feedback inhibition of PDH, as well as causing activation of PDH kinase and conversion of PDH to the inactive phosphorylated form.

The cellular concentration of Ca^{++} in the heart increases as the cardiac workload increases. PDHP phosphatase is activated by Ca^{++} which increases its association with the PDH complex (Pettit F *et al.*, 1972). Both PDH kinase and phosphatase require Mg^{++} but the phosphatase requires a 10 fold higher concentration of this ion for optimal activity (Thomas AP *et al.*, 1986). Activation of PDHP phosphatase results in increased PDH activity and production of acetyl-CoA for the citric acid cycle.

PDH is activated in response to adrenaline and cAMP, although whether this is due to phosphorylation by PKA is unclear. It is likely that this activation is to some extent due to increased Ca^{++} influx into the mitochondria by an α-adrenergic mechanism (Kessar P and Crompton M, 1981). The availability of alternative fuels inhibits the workload-stimulated increase in PDH activity, but does not overcome its activation by cAMP (Depre C *et al.*, 1993). In adipose tissue PDH is activated by insulin (Kruszynska YT and McCormack JG, 1989), whilst in heart no effects of insulin have been observed (Randle PJ *et al.*, 1978).

1.4.2. Fatty acids.

Fatty acid derived acetyl-CoA is produced in the mitochondria by the process of β-oxidation. Before this oxidation occurs cytosolic fatty acids must first be activated and transported across the mitochondrial membranes. These processes are achieved with the involvement of fatty acyl-CoA synthetases, carnitine palmitoyl transferases and carnitine acyltranslocase.

1.4.2.1. Fatty Acid Activation.

Fatty acyl-CoA synthetases catalyse the conversion of fatty acids into fatty acyl CoA derivatives in the reaction: ATP + fatty acid + CoASH $\leftrightarrow$ Acyl-CoA + AMP + PPi. The reaction is dependent on Mg^{2+} and can be stimulated by other cations particularly K^+. Enzymes specific for short, medium and long-chain fatty acids have been identified.

The subcellular location of fatty acyl-CoA synthetases varies between tissues. Medium chain fatty acids, the oxidation of which is independent of carnitine, are activated in the mitochondrial matrix (Aas M and Bremmer J, 1968). Palmitoyl-CoA synthetase is responsible for the activation of long chain fatty acids and in heart is located on the sarcoplasmic reticulum and the outer mitochondrial membrane (DeJong JW and Hülsman WC, 1970).

The manner in which fatty acyl-CoA synthetase is regulated is not clear with activity appearing to depend on substrate availability and product inhibition. It has been suggested that fatty acid activation is coupled to flux through the citric acid cycle by the carnitine-acetyl-CoA transferase (CAT) system, the proposed mechanism for this scheme is illustrated in figure 1.3. By shuttling excess acetyl-CoA units from the mitochondria the CAT enzymes decrease the cytosolic CoASH content and restrict fatty acyl-CoA synthetase activity(Oram JF *et al.*, 1975). As energy demand increases the acetyl-CoA:CoASH falls and fatty acid activation is stimulated. However it should be noted that this mechanism is dependent on the presence of a cytosolic CAT activity and evidence for the existence of such activity is lacking (Edwards YH *et al.*, 1974; Abbas AS *et al.*, 1998).

In heart palmitoyl-CoA synthetase is inhibited by palmitoyl-CoA, which causes an increase in the Km for CoASH (Pande SV, 1973), and this may provide a

mechanism by which fatty acid activation can be matched to rates of β-oxidation. Increasing the supply of exogenous fatty acids to the been shown to result in an increase in acyl-CoA with a corresponding decrease in the CoASH substrate (Oram JF *et al.*, 1973). The decreased affinity for CoASH caused by acyl-CoA would serve to further amplify this feedback inhibition. However in a later study no change in acyl-CoA could be observed in cardiac myocytes incubated with palmitate (Hamilton C and Saggerson ED, 2000). AMP also inhibits palmitoyl-CoA synthetase activity (Pande SV and Mead JF, 1968), and this may limit the accumulation of long-chain acyl-CoA derivatives during oxygen deprivation.

1.4.2.2. Fatty Acid Translocation.

Once long-chain fatty acids are activated they are unable to directly cross the inner mitochondrial membrane without first being transferred to carnitine. This reaction: acyl CoA + carnitine ↔ acylcarnitine + CoASH is catalysed by a carnitine-acyl CoA transferase. Long chain fatty acyl-CoAs are the substrate of carnitine palmitoyl transferase 1 (CPT1) the catalytic site of which is located on the outer side of the outer mitochondrial membrane (Fraser F *et al.*, 1997). In heart the oxidation of long chain fatty acids is dependent on carnitine. Because short and medium chain fatty acids can be activated in the mitochondrial matrix their oxidation is carnitine independent. Acylcarnitine produced by the CPT1 reaction is then a substrate for a carnitine:acylcarnitine translocase which facilitates its transport across the inner mitochondrial membrane. A second transferase (CPT2) is associated with the inner surface of the inner mitochondrial membrane and catalyses the regeneration of long chain acyl-CoA and carnitine inside the mitochondrial matrix. These reactions ensure that the mitochondrial and cytosolic pools of CoASH remain spatially separated and that the carnitine content on either side of the mitochondrial membrane is kept constant.

1.4.2.2.1. Regulation of CPT1.

The CPT1 reaction is the rate-controlling reaction of long-chain fatty acid translocation into the mitochondrial matrix (Eaton S *et al.*, 2001). Two isoforms of CPT1 exist, liver-type (L-CPT1) and skeletal muscle-type (M-CPT1) (Esser V *et al.*, 1996). The two isoforms vary in their kinetic

Figure 1.3. Control of Fatty acid Activation and Translocation.

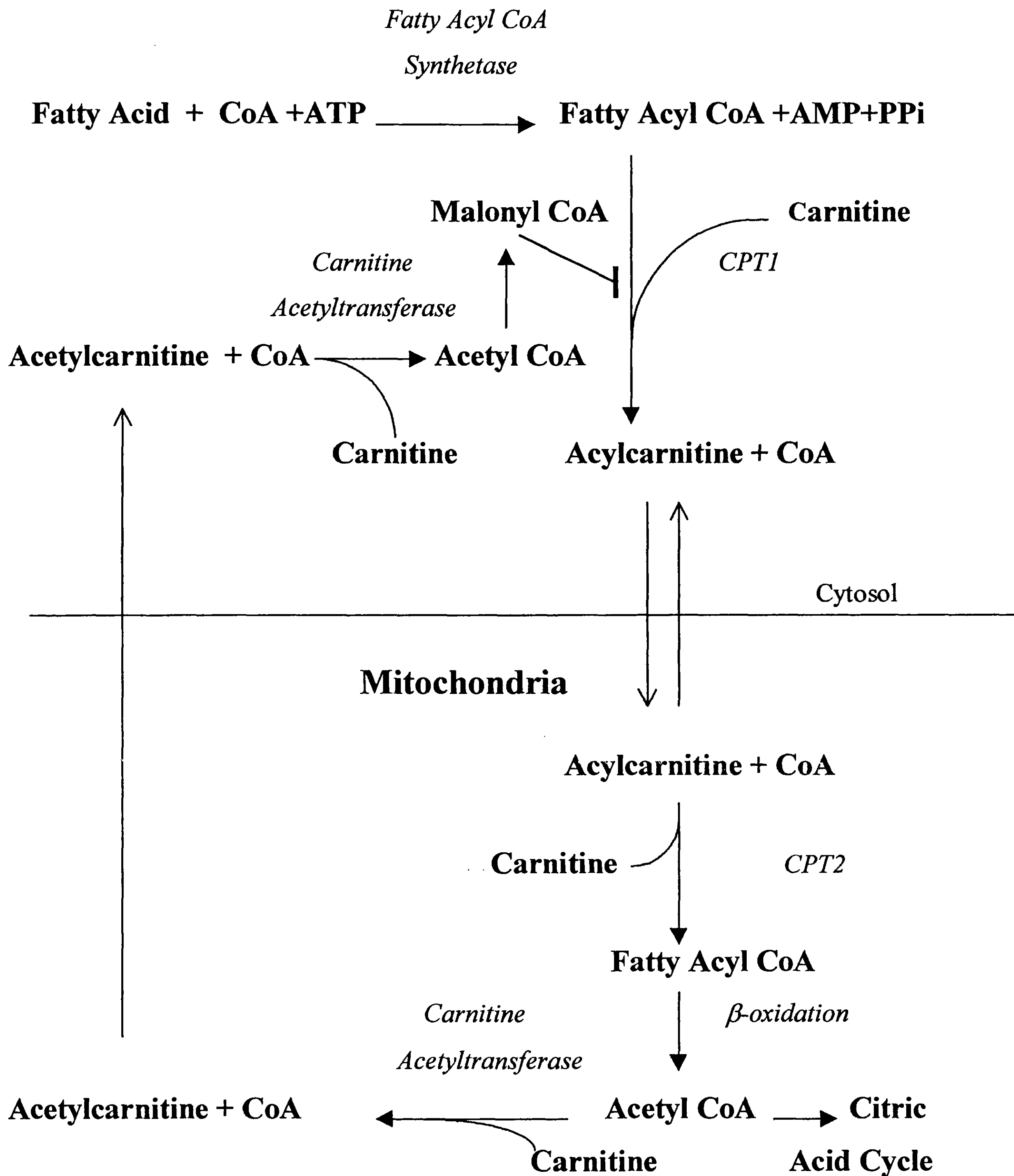


Figure 1.3. Excess mitochondrial acetyl CoA can be transported into the cytosol as acetylcarnitine. This increases the cytosolic acetyl CoA:CoA, inhibiting fatty acid activation.

parameters, with L-CPT1 having a higher affinity for carnitine and although both isoforms are inhibited by malonyl-CoA, the liver form is less sensitive to inhibition by this effector (Saggerson ED and Carpenter CA, 1981). Heart CPT1 displays characteristics intermediate to CPT1 from liver and skeletal muscle (McGarry JD *et al.*, 1983). This observation is explained by the discovery that adult heart muscle contains both CPT1 isoforms with M-CPT1 predominating (Weis BC *et al.*, 1994). The sensitivity of liver CPT1 to inhibition by malonyl-CoA can be decreased under certain conditions including fasting (Cook GA, 1984) and diabetes (Cook GA and Gamble MS, 1987). In the heart no change in the malonyl-CoA sensitivity of CPT1 has been observed in response to dietary and hormonal alterations (Cook GA and Lappi MD, 1992). Control of fatty acid translocation and oxidation under such conditions in this tissue appears to be due primarily to changes in the concentration of malonyl-CoA.

1.4.2.3. β-Oxidation.

Fatty acyl-CoAs delivered to the mitochondrial matrix are converted to acetyl-CoA by the reactions of the β-oxidation pathway. This process comprises four reactions:

1. Acyl-CoA + FAD $\rightarrow$ Δ^2–3-*trans*-enoyl-CoA + $FADH_2$
2. Δ^2–3-*trans*-enoyl-CoA + H_2O $\rightarrow$ L-3- Hydroxyacyl- CoA
3. L-3- Hydroxyacyl-CoA + NAD^+ $\rightarrow$ L-3- Ketoacyl-CoA + NADH + H^+
4. L-3- Ketoacyl-CoA + CoASH $\rightarrow$ Acyl-CoA (- 2 carbons) + Acetyl-CoA

These reactions are catalysed by acyl-CoA dehydrogenases, enoyl-CoA-hydratase, L-3-hydroxyacyl-CoA dehydrogenase and 3-oxoacyl-CoA thiolase respectively. These enzymes are found in the mitochondrial matrix in close association with the inner membrane (Beattie DS, 1968). Fatty acids of chain length C_4 to C_{20} are oxidized by this series of reactions with acyl-CoA dehydrogenase enzymes specific for short, medium and long-chain fatty acids existing.

Factors controlling cardiac β-oxidation include the availability of fatty acyl-CoA, NAD^+ and FAD, the concentration of high energy phosphates and the

availability of CoASH. Under normoxic conditions the transport of acyl units across the inner mitochondrial membrane is a major control factor. The rate of β-oxidation is regulated to match the flux through the citric acid cycle. When energy demand is low rates of fatty acid oxidation decrease due to an increase in the mitochondrial $NADH/NAD^+$ ratio. A high $NADH:NAD^+$ is associated with an accumulation of L-3-hydroxyacyl-CoA due to inhibition the L-3-hydroxyacyl-CoA dehydrogenase reaction (Bremer J and Wojtczak AB, 1972). In hearts subjected to increased workload there is an increase in flux through the citric acid cycle, a decrease in $NADH:NAD^+$ and a decrease in acetyl-CoA:CoASH. The decrease in the mitochondrial acetyl-CoA/CoASH ratio causes an activation of 3-oxoacyl-CoA thiolase and increases provision of acetyl-CoA for the citric acid cycle.

1.4.3. Citric acid cycle.

Under aerobic conditions acetyl-CoA derived from glucose or fatty acids is completely oxidized to CO_2 by the mitochondrial enzymes of the citric acid cycle. This series of reactions generates 2 molecules of CO_2, 3 NADH and 1 $FADH_2$. NADH and $FADH_2$ produced during glycolysis, β-oxidation and the citric acid cycle are oxidized in the presence of molecular O_2 by the electron transport chain to drive ATP synthesis and regenerate NAD^+ and FAD. Although molecular O_2 is not directly involved in the enzymatic reactions, the citric acid cycle can operate only under aerobic conditions as O_2 is necessary for the regeneration of the NAD^+ and FAD cofactors.

The rate of flux through the citric acid cycle is adjusted to meet the cellular ATP requirements. The most crucial regulators of cycle activity are its substrates acetyl-CoA, oxaloacetate and NAD^+. Acetyl-CoA and oxaloacetate are present in mitochondria at concentrations below the Km for citrate synthase (LaNoue K *et al.*, 1970), and changes in their levels result in an altered rate of this reaction. Production of acetyl-CoA from pyruvate and fatty acids is controlled by PDH and the β-oxidation reactions respectively. When hearts are perfused with octanoate acetyl-CoA for citrate synthase is derived from β-oxidation, a situation which favours pyruvate carboxylation (Comte B *et al.*, 1997). Under these conditions the high availability of both acetyl-CoA and oxaloacetate favours the formation of citrate. Oxaloacetate can also be formed from malate, the concentrations of both vary according to the

NADH:NAD$^+$. Under conditions where intramitochondrial NADH decreases there is increased conversion of malate to oxaloacetate and greater flux through the citrate synthase reaction (Bowman RH, 1966). Aspartate transamination is also an important mechanism for the generation of oxaloacetate.

Two other mechanisms are involved in regulating citric acid cycle activity. Firstly the redox state of FAD and NAD$^+$ and secondly the allosteric control of citrate synthase and dehydrogenase reactions (LaNoue K *et al.*, 1970). Four reactions require NAD$^+$ or FAD, the concentrations of which fall when the cellular energy status is high. High ATP levels reduce the activity of citrate synthase by increasing the Km for acetyl-CoA, and also inhibit the isocitrate dehydrogenase reaction. Isocitrate dehydrogenase is inhibited by NADH and stimulated by ADP which decreases the Km for the reaction substrates isocitrate and NAD$^+$. α-ketoglutarate dehydrogenase is inhibited by its reaction products succinyl-CoA, an effect enhanced by NADH. Both isocitrate dehydrogenase and α-ketoglutarate dehydrogenase can be activated by increases in mitochondrial Ca^{++} concentration (McCormack JG *et al.*, 1990).

1.5. Interaction of Carbohydrate and Fatty Acid Utilisation.

The relative contribution of fatty acids and glucose to myocardial ATP production can be altered in response to fuel availability, cardiac workload and upon hormonal stimulation. Increased availability of fatty acids which occur during fat feeding, fasting and diabetes result in decreased utilisation of carbohydrate fuels, the 'glucose-fatty acid cycle' (Randle P.J. *et al.*, 1963) and a decreased sensitivity of the heart to insulin. There is also evidence that carbohydrate fuels can regulate fatty acid oxidation. The mechanism by which this effect is exerted remains to be elucidated although a role for the metabolite malonyl-CoA has been proposed.

The aim of the following section is to outline the mechanisms by which lipid fuels exert their glucose sparing effect, and to describe the contribution of each substrate under basal and hormone stimulated conditions.

1.5.1. Normoxic Metabolism.

When oxygen supply is adequate fatty acids are the preferred substrate of the cardiomyocyte. Addition of fatty acids to heart perfusion medium decreases the oxidation of ^{14}C-labelled glucose and increases the amount of label recovered in tissue glycogen (Shipp JC *et al.*, 1961), indicating that fatty acids are selected for energy production whilst glucose is directed toward storage.

The employment of pulse-chase techniques in which myocardial TAG stores are ^{14}C- labelled during a pre-perfusion period, followed by exposure of the heart to ^{3}H labelled palmitate has allowed the relative contribution of exogenous and endogenous fatty acid to ATP production to be determined under a variety of perfusion conditions. Rates of glycolysis and glucose oxidation can be determined by perfusing hearts with 2- [^{3}H] and U-[^{14}C] glucose respectively, due to the production of $^{3}H_2O$ at the phosphoglucoisomerase step of glycolysis and the liberation of $^{14}CO_2$ resulting from PDH and citric acid cycle activity.

Saddik and Lopaschuk, (1991) utilized these techniques to elucidate the contribution of substrates to ATP production under perfusion conditions of 11mM glucose (no fat), and 11mM glucose plus either 0.4mM palmitate (low fat) or 1.2mM palmitate (high fat). The results of this study showed that as the exogenous fatty acid supply increased the contribution of glucose to ATP production decreased from over 40% (no fat) to around 8% in the high fat group. Endogenous fatty acids were oxidized to provide over 50% of the heart's ATP requirements in the absence of exogenously provided fatty acid, a figure which decreased to 11% in the high fat perfusions. The same study showed that glucose oxidation was more sensitive to inhibition by fatty acids than glycolysis, with production of $^{14}CO_2$ falling 10 fold between the no fat and high fat perfusions but only a 50% decrease in the production of $^{3}H_20$.

Fatty acids exert their inhibitory effect on glucose utilisation at the level of glucose uptake, PFK1 and PDH (figure 1.4). Oxidation of fatty acids is necessary for their negative effect on glucose utilization, with inhibition being lost under anoxic conditions. The mechanism by which glucose uptake is reduced by fatty acids is not fully elucidated, although the accumulation of intracellular glucose measured in hearts perfused with fatty acid (and also

Figure 1.4 Fatty Acid Inhibition of Glucose Utilisation.

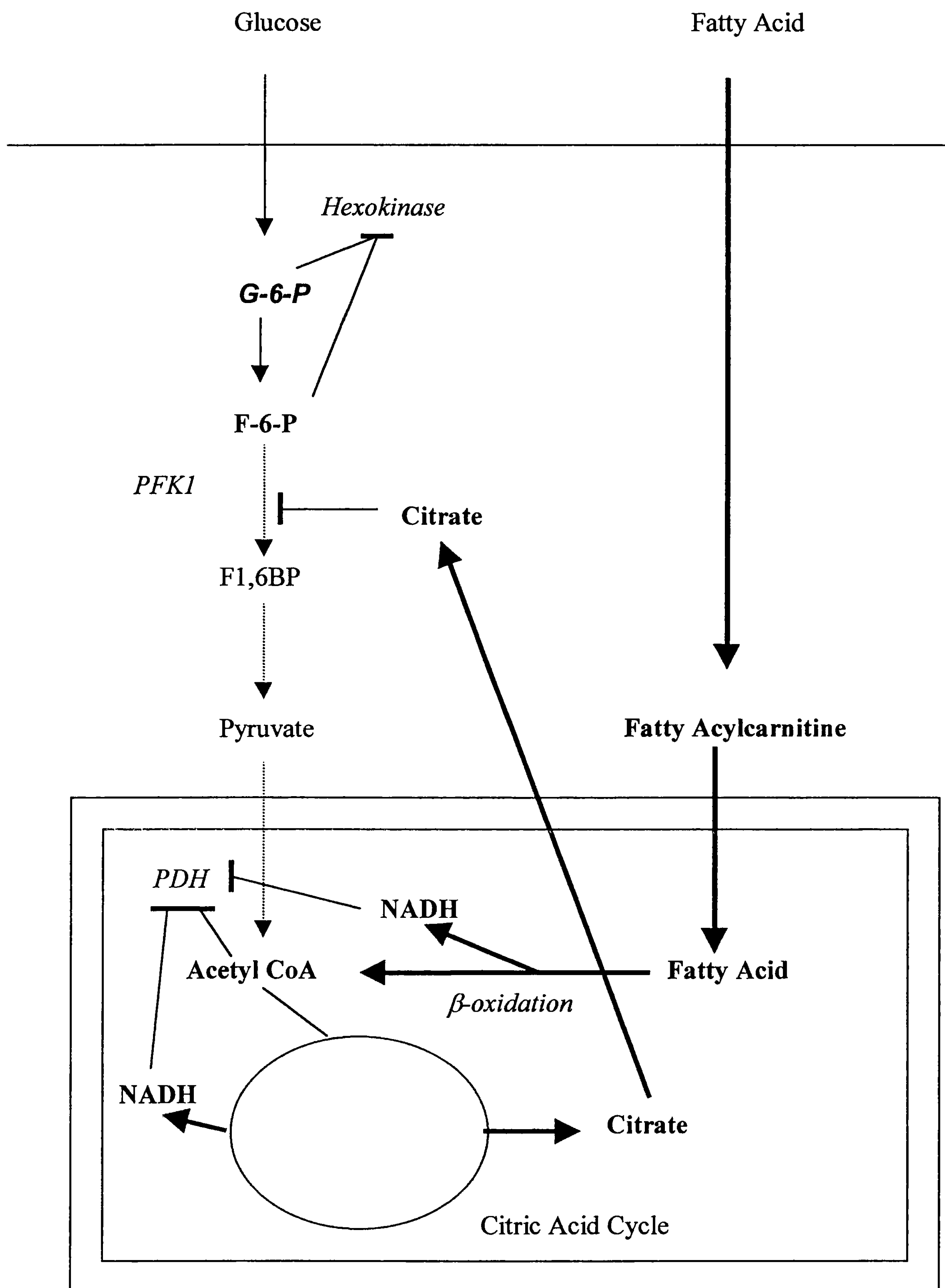


Figure 1.4 Acetyl-CoA and NADH produced by ß-oxidation and the citric acid cycle are able to inhibit glucose oxidation via PDH inactivation. PFK activity is reduced by citrate, the resulting accumulation of G6P and F6P is able to inhibit HK.

ketone bodies and pyruvate) indicate that glucose phosphorylation is inhibited (Randle P.J. *et al.*, 1963). Rates of glucose transport are dependent on the level of circulating insulin and the tissue sensitivity to this hormone. Under conditions where circulating fatty acids are elevated, such as during fasting, the glucose sparing effect of fatty acids is supported by low plasma insulin levels. High concentrations of circulating fatty acids can also decrease the sensitivity of heart tissue to insulin stimulation (Neely JR *et al.*, 1969).

The generation of acetyl-CoA from fatty acids results in the production of NADH both from β-oxidation and the citric acid cycle. As a consequence the mitochondrial NADH/NAD^+ ratio is increased with the result that the dehydrogenase reactions of the citric acid cycle are inhibited (LaNoue K *et al.*, 1970). With continued production of acetyl-CoA from β-oxidation there is an unspanning of the cycle with the rate of citrate synthesis exceeding flux through the later reactions. In this way, perfusion of rat hearts with fatty acids results in an accumulation of the citric acid cycle intermediates citrate, isocitrate, α-ketoglutarate and malate (Bowman RH, 1966). Excess citrate signals high substrate availability and is able to enter the cell cytosol via a mitochondrial tricarboxylate transporter (Vincent G *et al.*, 2000). This rise in cytosolic citrate is able to inhibit both PFK1 and PFK2 directly, and PFK1 indirectly due to the decreased production of F2,6BP. Inhibition of PFK1 results in an accumulation of G-6-P and F-6-P and feedback inhibition of HK and stimulation of glycogen synthesis.

The most pronounced effect of fatty acids is seen at the level of PDH and results in the almost total inhibition of glucose oxidation. PDH is inhibited by its products acetyl-CoA and NADH, both of which increase when hearts are perfused with fatty acids. PDH kinase is stimulated by these molecules, and results in the phosphorylation and further inhibition of PDH (Kerbey AL *et al.*, 1976).

1.5.2. Effects of Hormones.

1.5.2.1. Insulin.

Insulin is secreted by the β- cells of the pancreas in response to elevated blood glucose and therefore exposure of tissues to the hormone signals the

fed state. Treatment of cells with insulin stimulates glucose transport, glycogen synthesis as well as increasing glucose utilisation.

The metabolic effects of insulin are initiated by its binding to a receptor in the plasma membrane. This receptor is a tyrosine kinase which becomes activated by binding of the hormone and initiates a complex series of phosphorylation/ dephosphorylation reactions, involving the activation of the mitogen-activated protein (MAP) kinase and phosphatidylinositol 3-kinase (PI 3-kinase) signalling pathways. PI 3-kinase catalyses the formation of phosphatidylinositol (3,4,5) triphosphate, a key second messenger in insulin signalling which is involved in the activation of downstream targets of the hormone such as protein kinase B (PKB) and the p70 ribosomal S6 kinase (p70 S6K) (Shepherd PR *et al.*, 1998). Insulin-mediated dephosphorylation of its substrates is proposed to be due to activation of protein phosphatases, and evidence exists for increased activity of PP1 in cultured cells in response to insulin treatment (Chan CP *et al.*, 1988).

Insulin stimulation of glucose transport is due to increased translocation of GLUT4 transporters to the plasma membrane from intracellular storage vesicles. The protein phosphatase inhibitor okadaic acid is able to mimic the effect of insulin on glucose transport in muscle (Tanti JF *et al.*, 1991) and adipose tissue (Lawrence JC *et al.*, 1990), implying that an insulin stimulated phosphorylation event promotes GLUT4 recruitment. This effect is indirect in that the phosphorylation state of the glucose transporter itself is unchanged.

Inclusion of insulin in heart perfusion media containing 5mM glucose and 0.4mM palmitate results in increased rates of glucose oxidation (Sakamoto J *et al.*, 2000), an effect which was overcome by increasing the concentration of palmitate to 1.2mM (with 20mM glucose). Insulin has also been shown to enhance glycogen synthesis and inhibit glycogenolysis in perfused rat hearts (Goodwin GW *et al.*, 1995). These effects are due to insulin modulation of the activities of glycogen synthase and phosphorylase and PFK2.

Insulin causes dephosphorylation and activation of glycogen synthase, an effect mediated by the inactivation of glycogen synthase kinase-3, as well as dephosphorylation and inactivation of glycogen phosphorylase. Taken together with the hormone stimulation of glucose uptake these two dephosphorylation events explain the increased tissue glycogen content of

tissues exposed to insulin. Insulin activates PFK2 by increasing its phosphorylation, an event proposed to be mediated by activation of PKB (Deprez J *et al.*, 1997). This activation results in an increase in the tissue content of F2,6BP which causes stimulation of PFK1 and increased glycolytic flux.

1.5.2.2. Adrenaline.

The inclusion of catecholamines in heart perfusion media results in an increase in the frequency and force of contraction and an increase in ATP utilisation. Under these conditions there is increased oxidative phosphorylation, increased utilisation of acetyl-CoA and citric acid cycle flux, and lower levels of NADH. Various studies have been undertaken to determine which metabolic pathways are activated to meet this increased energy demand, the conclusions of which are summarised in figure 1.5.

A study in 1966 by Kreisberg showed that the increased energy requirements elicited by adrenaline in rat hearts perfused with a constant fatty acid concentration were primarily met by increased glucose oxidation. This study also showed that whilst endogenous lipid turnover was accelerated, in contrast with earlier studies (Goutis A and Felts JM, 1963) adrenaline was unable to stimulate the uptake of exogenous fatty acids. The effect of catecholamines on TAG turnover is most likely mediated by increases in cAMP and PKA stimulation of cardiac lipases, particularly HSL.

Later studies have evaluated the contribution of glycolysis, glucose and fatty acid oxidation rates to ATP production after adrenergic stimulation in perfused, working rat hearts (Collins-Nakai RL *et al.*, 1994). ATP production increased by 50% in response to adrenaline treatment, and this was associated with increases in both glycolysis and glucose oxidation (272% and 410% respectively), and a moderate (10%) increase in fatty acid oxidation rates. The increased rate of glucose oxidation seen with adrenaline was associated with an increase in PDH activity. This effect is likely to be due to an α-adrenergic stimulated increase in Ca^{++} and activation of PDH phosphatase. PDH is also activated by cAMP analogues by an unknown mechanism (Depre C *et al.*, 1998), an observation which implies a role for β-adrenergic pathways in the catecholamine induced stimulation of glucose oxidation. The increased glycolytic rate measured in response to adrenaline

Figure1.5 Adrenaline Stimulates Glucose Utilisation.

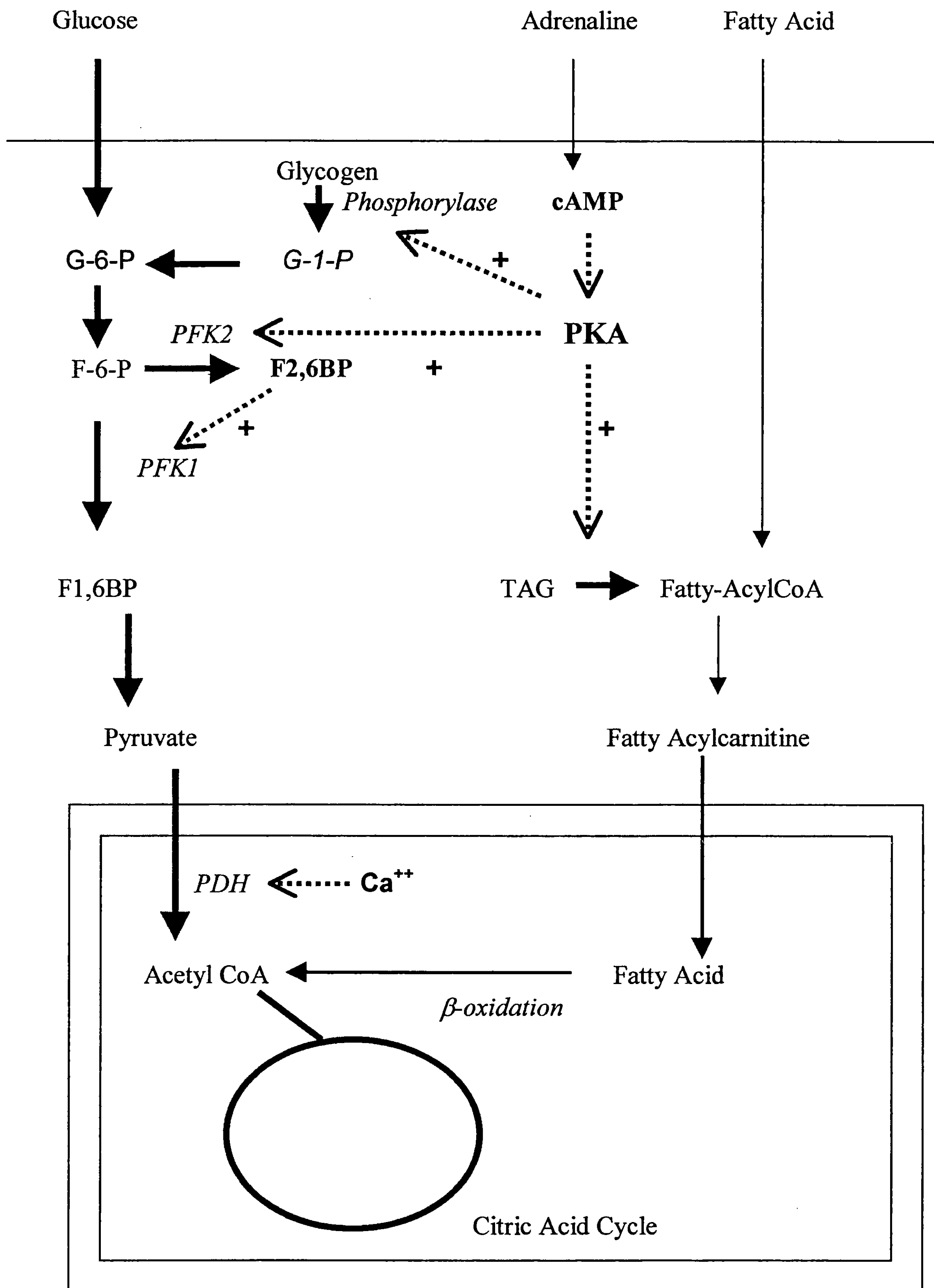


Figure 1.5 Adrenaline increases glucose uptake, glycogenolysis, glycolysis and glucose oxidation by PKA stimulation of phosphorylase & PFK2. PDH is activated by increases in Ca^{++}. Lipolysis is enhanced by adrenaline although total fatty acid oxidation is only slightly stimulated.

could be due to direct stimulation of PFK1 by Ca^{++}, or by activation of PFK2 via PKA phosphorylation. Accelerated flux through the citric acid cycle after addition of isoproterenol to heart perfusion media results in decreased citrate efflux (Vincent G *et al.*, 2000), which could relieve citrate inhibition of both PFK1 and PFK2.

Goodwin, Taylor and Taegtmeyer, 1998, confirmed the importance of carbohydrate utilisation in response to adrenaline. These authors showed that the initial increase in ATP demand was met by glycogenolysis, with glycogen being preferentially channelled toward oxidation. The uptake of glucose from perfusion media was increased upon prolonged stimulation. This delay in increasing glucose uptake prevents the investment of ATP to glucose phosphorylation when the cellular energy charge is low. Adrenaline increased the amount of phosphorylase in the active form due to the predicted stimulation of phosphorylase kinase by PKA and Ca^{++}. Levels of intracellular glucose and AMP also increased after adrenergic stimulation. This led the authors to predict that activation of phosphorylase b by AMP rather than stimulation of phosphorylase kinase is the important regulatory factor in the control of glycogenolysis under these conditions, as phosphorylase a is inhibited by high concentrations of glucose. Increases in the tissue concentration of AMP could also be responsible for accelerating glycolytic rates by allosteric activation of PFK1. Total β-oxidation was increased in this study by 40%, although again exogenous fatty acid utilisation was unaltered.

1.6. Malonyl-CoA Metabolism.

In certain circumstances carbohydrate fuels can inhibit the utilisation of fatty acids by cardiac tissue, although the mechanism by which this occurs is not completely understood the observation that carbohydrates are able to inhibit oleate but not octanoate oxidation suggest a role for CPT1 in the regulation of cardiac fuel selection. In non-lipogenic tissues such as heart and skeletal muscle malonyl-CoA has been proposed to act as a signalling molecule involved in the regulation of fatty acid oxidation. The muscle isoform of CPT1

is highly sensitive to malonyl-CoA inhibition and rates of fatty acid oxidation in heart can be correlated with malonyl-CoA levels under a variety of conditions (Saddik M *et al.*, 1993). Fatty acid utilisation increases in the heart following birth and this is associated with decreased tissue content of malonyl-CoA (Lopaschuk GD *et al.*, 1994). In rat hearts perfused with glucose as the sole substrate the addition of insulin results in an increase in malonyl-CoA, an effect which is overcome by palmitate and adrenaline (Awan MM and Saggerson ED, 1993). Altering the supply of glucose to isolated cardiac myocytes results in rapid changes in levels of malonyl-CoA (Hamilton C and Saggerson ED, 2000). Together these results indicate that heart cells contain enzymes which are able to respond rapidly to hormonal and nutritional signals and alter rates of fatty acid oxidation accordingly.

The tissue concentration of malonyl-CoA is a function of the relative rate of its synthesis and/or degradation. Although a role for the mitochondrial enzyme propionyl-CoA carboxylase cannot be totally ruled out it is widely accepted that the main enzyme responsible for the synthesis of malonyl-CoA in heart is acetyl- CoA carboxylase. Less is known about the mechanism by which malonyl-CoA is disposed. In heart the activity of fatty acid synthase is low (Awan MM and Saggerson ED, 1993) and fatty acyl-CoA elongation activity cannot be detected (Hamilton C and Saggerson ED, 2000), these mechanisms are unlikely to provide a route for the rapid disposal of malonyl-CoA in this tissue. A cardiac malonyl-CoA decarboxylase has been detected in heart muscle (Dyck JRB *et al.*, 1998) and a role for this enzyme in malonyl-CoA degradation has been proposed.

In the following section the mechanisms by which acetyl-CoA carboxylase and malonyl-CoA decarboxylase are regulated will be outlined and the role of the AMP-activated protein kinase cascade in the regulation of malonyl-CoA levels will also be discussed.

1.6.1. Acetyl- CoA Carboxylase.

Acetyl-CoA carboxylase (ACC) catalyses the first committed reaction in fatty acid biosynthesis, the carboxylation of acetyl-CoA to form malonyl-CoA. The overall reaction is a two step process involving the ATP-Mg^{++}-dependent formation of a carboxy-enzyme intermediate, followed by transfer of the carboxyl moiety to acetyl-CoA:

1. ATP + HCO_3^- + ACC. Biotin ⟶ ACC. Biotin.CO_2^- + ADP + Pi
2. ACC. Biotin.CO_2^- + acetyl-CoA ⟶ ACC. Biotin + malonyl-CoA

As well as its role as a precursor of fatty acid biosynthesis, malonyl-CoA also plays a role in the regulation of fatty acid oxidation by its inhibition of carnitine palmitoyltransferase I (section 1.4.2.2.1.). In muscle cells the primary role of ACC appears to be in regulating fatty acid oxidation (Saddik M *et al.*, 1993). This is supported by the fact that ACC is present in heart and skeletal muscle, despite the low fatty acid biosynthetic capacity of these tissues (Bianchi A *et al.*, 1990; Thampy KG, 1989).

1.6.1.1. ACC Isoforms.

In animal cells ACC exists as two isoforms with molecular weights of 265kDa (ACC1) and 280kDa (ACC2). Both isoforms show similarity in catalytic and regulatory regions, and vary most at their N-terminus. ACC2 has an extended N-terminal domain which accounts for most of the difference in molecular weight between the two isoforms. This domain has been proposed to target ACC2 to the mitochondrial membrane which may be important for its catalytic function (Ha J *et al.*, 1996). Recently ACC 2 has been shown by immunofluorescence microscopy to be localised on the mitochondrial membrane in a variety of animal cells (Abu-Elheiga L *et al.*, 2000). The two isoforms also show slight differences in kinetic parameters, with ACC2 having a higher Km for acetyl-CoA and a decreased affinity for citrate (Bianchi A *et al.*, 1990).

The two ACC isoforms vary in their tissue distribution. ACC1 is the major form in lipogenic tissues such as lactating mammary gland and is the only isoform expressed by white adipose tissue. In tissues with very little fatty acid synthetic ability such as heart and skeletal muscle ACC2 is the principle isoform expressed. Both isoforms exist in significant amounts in liver. These observations have led to the proposal that ACC2 may be responsible for the regulation of fatty acid oxidation and ACC1 with regulation of fatty acid synthesis.

1.6.1.2. ACC Isoform Expression.

The half-life of ACC varies from between 1 and 3 days in rat liver, depending upon physiological conditions and so changes in the cellular concentration do

not occur rapidly (Nakanishi S and Numa S, 1970). Levels of ACC protein alter depending upon hormonal and nutritional signals. Rat mammary gland ACC activity and mRNA increase as the gland increases milk synthesis post-natally (Barber MC *et al.*, 1992), with a concomitant decrease in adipose tissue ACC expression. The same authors showed these changes to be due to the hormones prolactin and growth hormone. Glucose-6-phosphate stimulates the expression of ACC in the adipose tissue of rats weaned using a high carbohydrate diet, whereas rats weaned onto a high fat diet showed decreased ACC expression in both adipose tissue and liver (Foufelle F *et al.*, 1992). ACC expression is also stimulated in response to glucose in pancreatic β-cells (Brun T *et al.*, 1993).

Prolonged starvation results in decreased ACC activity in rat livers (Katsurada A *et al.*, 1990), which can be reversed by carbohydrate feeding. This reversal is correlated with increases in ACC mRNA levels. Insulin also plays a role in the regulation of ACC expression. Diabetic rats show low levels of ACC protein and mRNA in both liver and adipose tissue (Katsurada A *et al.*, 1990; Pape ME *et al.*, 1988), which can be reversed by insulin treatment.

Most studies of the regulation of ACC gene expression have focused on the ACC1 isoform and little is known about control of ACC2 expression. Key differences between heart and lipogenic tissues include observations that there is no alteration in the expression of ACC in diabetic rat hearts (Lopaschuk GD and Gamble J, 1994) and although changes in the expression of both isoforms occur in liver in response to fasting/refeeding, in heart and muscle no change in ACC2 is seen under the same conditions (Bianchi A *et al.*, 1990). Therefore although hormonal and nutritional signals are important for ACC1 expression, the role of these effectors in regulating heart ACC2 is as yet unclear.

1.6.1.3. Short-term Regulation of ACC.

ACC activity can be regulated in the short-term by allosteric effectors and reversible phosphorylation.

Citrate is an important feed-forward activator of ACC increasing its activity *in vitro* up to 50 fold (Hardie DG and Guy PS, 1980). However changes in

cellular citrate concentration do not always correspond to changes in ACC activity and the sensitivity of ACC to citrate can be altered by hormonally regulated covalent modification. The smallest form of ACC that exists *in vivo* is a dimer, known as the protomeric form, which when activated by citrate is polymerised into filaments, with the apparent molecular weight increasing to up to $8x10^6$ Da (Gregolin C *et al.*, 1966a). This polymerised form of the enzyme is highly active. Although loss of activity was observed under conditions leading to depolymerisation, these incubations contained inhibitors of ACC such as sodium and chloride ions or the reaction product malonyl-CoA (Gregolin C *et al.*, 1966b). Beatty and Lane, 1983 showed that ACC activation in response to citrate occurred prior to its polymerisation, and that the depolymerised ACC protomer was active. Later studies have confirmed that the dephosphorylated, protomeric form of ACC has substantial citrate-independent activity (Thampy KG and Wakil SJ, 1985). Control of ACC activity by allosteric effectors and phosphorylation are interactive processes. Polymerised ACC is resistant to inactivation by phosphorylation (Munday M.R. and Hemingway CJ, 1999). Pre-treatment of purified ACC with 10mM citrate caused a 75% reduction in its phosphorylation by AMPK compared to controls.

Long-chain fatty acyl-CoA esters are also inhibitory to the ACC reaction *in vitro*, (Thampy KG, 1989) their effect being overcome by citrate (Halestrap AP and Denton RM, 1974). However because the concentration of fatty acyl-CoA required for half-maximal inhibition is well below its cellular concentration the physiological relevance of this is not clear. Also, because free fatty acyl-CoAs in the cell exist bound to intracellular proteins, they may not be available for regulation of ACC *in vivo*. Glutamate has also been shown to activate ACC *in vitro*, an effect enhanced by, but not dependent on the presence of protein phosphatases and inhibited by prior ACC phosphorylation (Boone AN *et al.*, 2000).

The regulation of ACC in the short-term is mainly achieved by reversible phosphorylation and inactivation (Hardie DG and Cohen P., 1979). ACC activity in rat liver increases during the dark period (Davies SP *et al.*, 1992) when rats are feeding. Starvation causes ACC to become phosphorylated and inactivated in both liver (Thampy KG and Wakil SJ, 1988; Munday MR *et*

al., 1991) and mammary gland (Munday M.R. and Hardie DG, 1986), as does feeding a high fat diet (Davies SP *et al.*, 1992). The effects of starvation in rats can be overcome by re-feeding, with ACC activity increasing to levels above that in control fed animals (Munday MR *et al.*, 1991). Adrenaline and glucagon treatment of adipocytes (Holland R *et al.*, 1985; Brownsey RW *et al.*, 1979) and hepatocytes (Holland R *et al.*, 1984) causes an increase in ACC phosphorylation and a decrease in enzyme activity.

In streptozotocin diabetic rats the re-activation of ACC caused by re-feeding starved animals is not observed, suggesting that insulin plays a role in the regulation of ACC (Munday M.R. and Hardie DG, 1986). Insulin treatment of rat epididymal adipose tissue increases the amount of ACC in the polymerised form (Halestrap AP and Denton RM, 1974). Insulin has been shown to increase ACC activity in adipocytes (Haystead TAJ and Hardie DG, 1986) and Fao hepatoma cells (Witters LA and Kemp BE, 1992). When injected into rats insulin caused the dephosphorylation and activation of liver ACC (Mabrouk GM *et al.*, 1990). However in adipocytes insulin treatment resulted in an increase in ACC phosphorylation (Brownsey RW and Denton RM, 1982) at a site termed the 'I' site. Increased phosphorylation of ACC in response to insulin has since been observed in hepatocytes (Holland R and Hardie DG, 1985).

Whether phosphorylation at the 'I' site is the cause of the insulin-stimulated activation of ACC is not clear. Protein Phosphatase 1 has been shown to dephosphorylate the 'I' site. However in adipocytes treated with okadaic acid, an inhibitor of protein phosphatase 1, the effect of insulin to stimulate fatty acid synthesis was blocked (Haystead TAJ *et al.*, 1989). The insulin effect on ACC activity does not survive extensive purification and is not reversed by protein phosphatase treatment (Haystead TAJ and Hardie DG, 1986) despite the 'I' site being completely dephosphorylated. This led to the suggestion that the effect of insulin may be mediated by a low molecular weight effector which is removed during the purification process. The 'I' site has been identified as Ser 29 (Haystead TAJ *et al.*, 1988) a site phosphorylated by casein kinase II, without altering enzyme activity (Munday M.R. and Hardie DG, 1984). However casein kinase phosphorylation of ACC has been proposed to cause dephosphorylation of sites associated with inhibition of enzyme activity

(Sommercorn J *et al.*, 1987) and cause increased association of protein phosphatases with ACC (Krakower GR and Kim K.H, 1985). Insulin treatment of adipocytes and hepatocytes has been reported to cause the polymerisation of ACC as judged by size exclusion chromatography (Borthwick AC *et al.*, 1987) and provides another possible mechanism by which ACC activity is increased in response to this hormone.

Analysis of the sequence of purified ACC phosphopeptides has shown that the enzyme contains at least eight phosphorylation sites. Protein kinases acting at these sites include PKA, AMPK, calmodulin-dependent protein kinase II, protein kinase C and casein kinases I and II. However only phosphorylation by PKA and AMPK produce changes in ACC activity as well as causing a decreased sensitivity to citrate (Munday M.R. *et al.*, 1988). Phosphorylation of ACC by these two kinases is mutually exclusive, with phosphorylation by one preventing subsequent phosphorylation by the second (Munday M.R. *et al.*, 1988).

PKA phosphorylates two sites on ACC identified as Ser 77 and Ser 1200 in the rat sequence. Phosphorylation by PKA results in an inactivation of ACC with a small decrease in the Vmax and an increase in the Ka for citrate (Munday M.R. *et al.*, 1988). That inactivation is due to phosphorylation is confirmed by observations that the effect is blocked by a protein inhibitor of PKA and is reversed on treatment with protein phosphatases (Ingebritsen TS *et al.*, 1983). Other researchers were unable to show direct phosphorylation of purified rat liver ACC by PKA and have shown that the phosphorylation observed was due to direct action of a carboxylase kinase which was itself activated by PKA (Lent BA and Kim K.H, 1983). More recent evidence suggests that PKA does not directly phosphorylate ACC in intact cells, and that the enzyme is phosphorylated in response to adrenaline and glucagon at Ser 79, a site phosphorylated by AMPK in hepatocytes (Sim ATR and Hardie DG, 1988) and adipocytes (Haystead TAJ *et al.*, 1990).

The mechanism by which adrenaline and glucagon increase AMPK activity is not clear. In adipocytes the effect of adrenaline is mediated via β-receptors and therefore by production of cAMP (Haystead TAJ *et al.*, 1990). The effects of adrenaline on ACC activity are seen when PKA is directly activated by forskolin or cAMP analogues, indicating regulation of AMPK activity is

mediated by PKA. However, Davies *et al,* 1989 saw no direct effect of PKA on AMPK activity. Another explanation for the effect of adrenaline on ACC in adipocytes is that by inhibition of protein phosphatase 1 via PKA phosphorylation of Inhibitor 1, there is an activation of AMPK and a decrease in ACC activity due to the increased phosphorylation of both enzymes. The effect of glucagon on hepatic ACC cannot be so easily explained. Hepatocytes do not contain Inhibitor 1 and in liver protein phosphatase 2A and not protein phosphatase 1 has been shown to be responsible for ACC dephosphorylation (Ingebritsen TS *et al.*, 1983).

AMPK phosphorylates ACC at Ser 79, Ser 1200 and Ser 1215 in the rat ACC sequence, however Ser 1215 phosphorylation is not associated with changes in the enzyme's activity (Davies SP *et al.*, 1990; Ha J *et al.*, 1994). Phosphorylation of ACC at Ser 79 by AMPK results in a large decrease in Vmax (80-90%) and an increase in the Ka for citrate (Munday M.R. *et al.*, 1988).

Removal of the Ser 77 and Ser 79 containing N-terminus of ACC results in a full activation of the enzyme and has led to the theory that only these two sites are responsible for ACC regulation (Davies SP *et al.*, 1990). However mutation of Ser 79 in ACC1 expressed in HeLa cells not only abolished the inhibitory effect of AMPK but prevented AMPK phosphorylation of Ser 1200. Although the role of Ser 1200 in regulating ACC activity is not clear, interestingly it was mutation of this site (and not Ser 77) that abolished the inhibitory effect of PKA (Ha J *et al.*, 1994).

1.6.1.4. Regulation of ACC2.

ACC2 from rat liver is a better substrate for PKA than ACC1 (Winz R *et al.*, 1994), despite the absence of the Ser 1200 PKA phosphorylation site. The extended N-terminus of ACC2 contains PKA consensus sequences, which have been proposed to play a role in regulating the association of the enzyme with the mitochondrial membrane (Kim K.H, 1997), and phosphorylation of such sites may not produce activity changes measurable *in vitro*. AMPK phosphorylation of purified rat muscle ACC resulted in a decrease in Vmax and an increase in the Ka for citrate, as well as increases in the Km values for ATP, acetyl-CoA and bicarbonate (Winder WW *et al.*, 1997). The same authors showed that phosphorylation by PKA of ACC caused no change in

kinetic parameters. ACC2 phosphorylation increased rapidly when cardiac myocytes were incubated with isoprenaline, although again no change in enzyme activity was observed (Boone AN *et al.*, 1999). In contrast to ACC1 the major sites phosphorylated in response to adrenergic stimulation in this study were PKA sites, with AMPK sites being much less affected. Dyck *et al*, 1999, observed inhibition of the activity of ACC2 purified from rat heart after treatment with PKA. In the same study rat heart ACC2 was also inactivated by AMPK, although this occurred less rapidly than the inactivation by PKA. In view of these results it is possible that ACC2 from heart is uniquely regulated.

The major role of ACC in non-lipogenic tissue is proposed to be in the regulation of fatty acid oxidation. Rates of fatty acid oxidation in cardiac myocytes (Awan MM and Saggerson ED, 1993) and perfused rat heart (Saddik M *et al.*, 1993) decrease with increasing concentrations of malonyl-CoA, due to the high sensitivity of cardiac CPT1 to this effector. ACC2 deficient mice show decreased levels of malonyl-CoA and have increased rates of fatty acid oxidation (Abu-Elheiga L *et al.*, 2001). Rates of fatty acid oxidation increase in the heart following birth. This change in fuel utilization corresponds to decreases in both ACC activity and malonyl-CoA concentration in the developing rabbit heart (Lopaschuk GD *et al.*, 1994). Animals also rely on increased rates of fatty acid oxidation to support their energy requirements during hibernation, and decreased ACC activity and protein have been shown in hibernating compared to non-hibernating ground squirrels (Belke DD *et al.*, 1998).

Increases in fatty acid oxidation seen during the reperfusion of ischaemic hearts correlate with reduced malonyl-CoA levels. These decreases in malonyl-CoA are associated with decreased ACC activity and increases in the activity of AMPK (Kudo N *et al.*, 1995; Kudo N *et al.*, 1996). Exercise (Winder WW and Hardie DG, 1996) and electrical stimulation (Vavvas D *et al.*, 1997) of rat skeletal muscle cause decreases in malonyl-CoA and ACC activity. Again these changes correspond to increases in the activity of AMPK. Another study (Hutber CA *et al.*, 1997) showed decreases in malonyl-CoA and ACC activity in electrically stimulated muscle, and although AMPK activity was increased its activation was subsequent to the inhibition of ACC.

Unlike the situation for ACC1 in liver and mammary gland, fasting and refeeding have no effect on either ACC2 expression or activity in heart (Bianchi A *et al.*, 1990) or skeletal muscle (Winder WW *et al.*, 1995) although in this tissue a decrease in malonyl-CoA was measured. Later studies have also measured decreases in malonyl-CoA levels in fasted rat heart muscle (Abu-Elheiga L *et al.*, 2001).

Hormones such as insulin are able to regulate fatty acid oxidation by increasing the cellular concentration of malonyl-CoA in heart (Awan MM and Saggerson ED, 1993) and skeletal muscle (Saha AK *et al.*, 1997; Alam N and Saggerson ED, 1998). The mechanism by which insulin exerts its effect is not clear. Saha *et al*, 1997 found that in skeletal muscle levels of both citrate and malate increased in response to insulin, but no change in ACC activity was observed. ACC2 does not contain the Ser 29 site phosphorylated in ACC1 in response to insulin, and it has been proposed that insulin activates ACC2 allosterically by increasing levels of citrate rather than by altering the phosphorylation state of the enzyme. ACC2 has a lower affinity for acetyl-CoA than ACC1 and availability of substrate may be important for regulating the activity of this isoform. Kudo *et al*, 1995 found that decreases in malonyl-CoA content could be observed in heart at the end of ischaemia and preceded the inactivation of ACC which became significant only at the end of a reperfusion period. This result was explained by changes in the tissue concentration of acetyl-CoA, which was decreased during the ischaemic period, and suggest that this was the important factor in the control of malonyl-CoA production. In agreement with this is the observation by Saddik *et al*, 1993 that there is a significant correlation between acetyl-CoA and malonyl-CoA concentrations in perfused rat hearts. Increasing the concentration of acetyl-CoA by including the PDH activator DCA in the perfusion media resulted in increased levels of malonyl-CoA, whilst decreasing PDH activity by omitting glucose from the perfusate resulted in a marked decrease in malonyl-CoA. Increasing the cytosolic citrate concentration could potentially modulate the activity of ATP-citrate lyase, and increase provision of acetyl -CoA for ACC. Inhibition of ATP-citrate lyase by (-) –hydroxycitrate has been shown to overcome the insulin and glucose stimulated increase in malonyl-CoA in rat soleus muscle (Saha AK *et al.*, 1997).

1.6.2. Malonyl CoA Decarboxylase.

The prime candidate for the removal of malonyl-CoA in non-lipogenic tissues is malonyl-CoA decarboxylase (MCD), which catalyses the formation of acetyl -CoA from malonyl-CoA. This enzyme is present in the mitochondrial matrix in several tissues where it has been proposed to play a role in preventing the inhibition of mitochondrial carboxylases by malonyl-CoA. MCD activity is higher in tissues which have high fatty acid oxidation rates such as heart and liver, and lower in tissues such as brain which primarily utilise glucose (Voilley N *et al.*, 1999).

In order for MCD to function as a regulator of malonyl-CoA concentration and fatty acid oxidation rates it is necessary for it to have extra-mitochondrial activity. Two isoforms of MCD have been found in the goose uropygial gland, an organ that synthesises wax-type lipids (Kim YS and Kolattukudy PE, 1978). These isoforms are the product of a single gene, the use of two promoters allowing synthesis of a longer protein containing a mitochondrial target sequence, and a shorter cytosolic enzyme. The precise subcellular location of mammalian MCD remains unclear. Evidence for cytosolic decarboxylase activity that cannot be attributed to mitochondrial damage has been provided in skeletal muscle (Alam N and Saggerson ED, 1998). Isolated heart mitochondria have also been shown to contain a MCD activity that is overt (Hamilton C and Saggerson ED, 2000).

MCD expression in heart and skeletal muscle is sensitive to circulating fatty acid concentrations (Young ME *et al.*, 2001), and these authors provide evidence that MCD expression is controlled by fatty acids via peroxisome proliferator-activated receptor-α (PPARα). MCD mRNA in heart and muscle was shown to increase after 1 day of high fat feeding. Enzyme activity was increased by 30% after 4 days in soleus muscle whilst in heart a decrease in activity was observed. MCD mRNA levels in both tissues increased during a two day fast and returned to control levels after 4 days of re-feeding a low fat diet. No change in MCD activity was measured in response to fasting, although cardiac MCD activity was decreased upon refeeding. MCD mRNA and activity was increased in skeletal muscle in streptozotocin diabetic rats. No activity change was observed in diabetic rat hearts despite an increase in MCD mRNA. MCD activity is also increased in the liver of diabetic rats (Dyck

JRB *et al.*, 2000). Hypertrophied rat hearts show decreased rates of fatty acid utilization, and this is associated with a decrease in MCD expression and activity that can be prevented by feeding a low fat diet (Young ME *et al.*, 2001). A separate study (Chien D *et al.*, 2000) which reported increases in malonyl-CoA in skeletal muscle with re-feeding showed that these were not associated with alterations in either ACC or MCD activity.

The lack of correlation between MCD expression and enzyme activity suggests that post-translational mechanisms operate to regulate MCD activity. The activity of liver MCD increases during fasting, an effect not associated with protein concentration (Dyck JRB *et al.*, 2000), and these authors suggest that MCD may be regulated by phosphorylation/ dephosphorylation reactions. However in this study treatment of purified MCD *in vitro* with casein kinase II, AMPK, PKC or protein phosphatases 2A and 2C produced no change in enzyme activity. Other studies have shown different results on the effects of phosphorylation on MCD activity. Decreases in malonyl-CoA following contraction in skeletal muscle are associated with an inactivation of ACC and an activation of AMPK (Vavvas D *et al.*, 1997). A later study by the same group showed that MCD activity increased under these conditions and paralleled the activation of AMPK (Saha AK *et al.*, 2000). These authors showed that MCD activity could be stimulated by incubating skeletal muscle with the cell permeable activator of AMPK, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), an effect reversed by protein phosphatase 2A treatment. In contrast to this result Dyck *et al*, 1998 showed that treatment of heart tissue with alkaline phosphatase increased MCD activity, implying inhibition of MCD by phosphorylation, and MCD activity was not altered in heart following ischaemia/ reperfusion despite activation of AMPK. A later study has shown that skeletal muscle MCD is not a substrate for AMPK (Habinowski SA *et al.*, 2001). The role of AMPK in the regulation of MCD activity therefore remains unclear. Increased rates of fatty acid oxidation that occur when hearts are subjected to increased workload are associated with increased MCD activity (Goodwin GW and Taegtmeyer H, 1999) with no change in ACC activity being observed. No measurement of AMPK activity was made in this study.

1.6.3. AMP- Activated Protein Kinase.

The AMP-activated protein kinase (AMPK) is the mammalian member of a metabolite-sensing family of protein kinases, the role of which appears to be in controlling the response to changes in cellular energy status. The AMPK system is highly sensitive to alterations in the ATP/AMP ratio and becomes activated when energy levels fall. AMPK phosphorylation of target proteins serves to inhibit ATP consuming processes and stimulate pathways of ATP generation.

1.6.3.1. AMPK Isoforms.

AMPK is a heterotrimeric complex consisting of subunits designated α, β and γ. The 63kDa α-subunit is the catalytic subunit and contains the N-terminal kinase domain and a C-terminus which contributes to the AMP binding site. Two isoforms of the α-subunit have been identified, termed α-1 and α-2. These isoforms show differences in substrate preference and it is possible that they regulate specific downstream targets (Woods A *et al.*, 1996). Another difference between the two isoforms is that complexes containing $\alpha 2$ but not $\alpha 1$ can be localised in the nucleus of INS-1 cells (Salt I.P. *et al.*, 1998).

Two isoforms of the non-catalytic β-subunit (38kDa) and three γ (35kDa) subunits have been described. The β subunit is myristoylated at its N-terminus and can be phosphorylated at several sites, although the significance of this phosphorylation is unclear (Mitchelhill K.I. *et al.*, 1997). The γ subunit shows no evidence of post-translational modification.

The function of the β and γ subunits is unclear, although expression of all three subunits is required for kinase activity. Interactions between β subunits and both α and γ subunits have been demonstrated in both reticulocyte lysates and using the yeast two-hybrid system, but no interaction was observed between α and γ subunits. These results have led to the suggestion that the β subunit mediates the formation of the heterotrimeric complex. Complexes containing the α-2 subunit have increased AMP dependency and are stimulated 4-6 fold by AMP, compared to a 2 fold increase in activity observed in α-1 containing heterotrimers (Salt I.P. *et al.*, 1998). The α-1 isoform is also more resistant to inactivation by PP2A, whilst both isoforms respond similarly to PP2C. The β-1 subunit predominates in

liver, with an equal mix of α-1 and α-2 catalytic subunits. The α-1 and β-1 subunits are widely distributed whilst the expression of the α-2 subunit is highest in liver, heart and skeletal muscle (Verhoeven AJM *et al.*, 1995), and β-2 is highly expressed in skeletal muscle. γ-1 and γ-2 isoforms are expressed widely whereas γ-3 mRNA is found only in skeletal muscle (Cheung PCF *et al.*, 2000). The AMP dependence of the heterotrimer is related to the γ isoform present, complexes containing γ-2 have the greatest dependence on AMP and those with γ-3 are the least AMP dependent.

1.6.3.2. AMPK Regulation.

AMPK activity is tightly regulated within the cell. The kinase is allosterically activated up to 5 fold by micromolar concentrations of AMP, an effect antagonised by ATP (Corton JM *et al.*, 1994). At a physiological concentration of ATP (4mM) the concentration of AMP required for half-maximal activation of AMPK is 30μM. AMPK is also regulated by phosphorylation by an upstream AMPK kinase (AMPKK). This upstream kinase is itself allosterically activated by AMP (Corton JM *et al.*, 1995). Palmitoyl-CoA has also been reported to stimulate a crude preparation of AMPKK (Carling D *et al.*, 1987; Davies SP *et al.*, 1989). There is no evidence that the upstream kinase is regulated by phosphorylation (Hawley SA *et al.*, 1996).

As well as allosteric activation, the binding of AMP to AMPK promotes its phosphorylation by AMPKK and makes it resistant to dephosphorylation by protein phosphatases (Davies SP *et al.*, 1995). The major site phosphorylated by AMPKK is the thr-172 residue within the α subunit of AMPK (Hawley SA *et al.*, 1996). As the sequence surrounding thr-172 is conserved in both α-1 and α-2 catalytic subunits it is likely that both isoforms are equally susceptible to phosphorylation at this residue. A higher AMP dependence has been observed in AMPK mutants lacking the thr-172 site (Stein SC *et al.*, 2000), raising the possibility that thr-172 phosphorylation is able to alter the sensitivity of AMPK to AMP. AMPK can be dephosphorylated *in vitro* by PP2A and PP2C. In intact rat hepatocytes AMPK dephosphorylation is not sensitive to okadaic acid indicating that PP2C is the physiologically active AMPK phosphatase (Moore F *et al.*, 1991).

High levels of ATP can antagonise the AMP-induced activation of the kinase cascade by competitively binding at the AMP binding site. ATP exerts its inhibitory effect in three ways. Firstly it prevents the allosteric activation of AMPK, it overrides the inhibition of PP2C dephosphorylation due to AMP and lastly it prevents phosphorylation of AMPK by AMPKK (Corton JM *et al.*, 1995; Davies SP *et al.*, 1995). These observations suggest that the AMPK cascade is sensitive to changes in the ratio ATP/AMP rather than AMP levels alone. Physiological concentrations of phosphocreatine have also been shown to allosterically inhibit AMPK (Ponticos M *et al.*, 1998) and as levels of this compound fall during contraction, phosphocreatine rather than AMP may be the principle regulator of AMPK in muscle.

The multiple effects of AMP on the AMPK cascade make it extremely sensitive to alterations in the concentration of this effector, a small increase in AMP being amplified by the system to produce a significant activation of AMPK. In intact cells ATP and AMP vary in a reciprocal manner due to the presence of adenylate kinase which acts to maintain the reaction 2ADP↔ATP + AMP close to equilibrium (Hardie DG *et al.*, 1998). If ATP production is inhibited the equilibrium is displaced and the ratio AMP/ATP increases and the AMPK cascade is stimulated.

1.6.3.3. Physiological Roles.

AMPK plays a key role in the regulation of carbohydrate and lipid metabolism. Downstream targets of the kinase include 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) which catalyses the regulatory step in sterol synthesis, ACC and HSL. Other substrates phosphorylated by AMPK *in vitro* include glycogen synthase, phosphorylase kinase (Carling D and Hardie DG, 1989), glycerol-3-phosphate acyltransferase (Muoio DM *et al.*, 1999), nitric oxide synthase (Chen Z-P *et al.*, 1999) and creatine kinase (Ponticos M *et al.*, 1998) but whether these enzymes are regulated *in vivo* remains to be confirmed.

ACC and HMG-CoA reductase were the first substrates of AMPK to be identified. AMPK phosphorylates ACC1 at three sites (section 1.6.1.3.) and HMG-CoA reductase is inhibited by AMPK phosphorylation at ser-871 (Clarke PR and Hardie DG, 1990). Incubation of rat hepatocytes with fructose causes a transient decrease in ATP levels and activation of AMPK which is

associated with inhibition of both ACC (Moore F *et al.*, 1991) and HMG-CoA reductase. ACC is phosphorylated and inhibited in Fao hepatoma cells in response to agents which inhibit oxidative phosphorylation (Witters LA *et al.*, 1991). These results indicate that the AMPK cascade serves to spare ATP by inhibiting the energy consuming pathways of fatty acid and cholesterol synthesis when the cellular energy charge is low. Although AMPK is regarded as the most important kinase involved in the regulation of liver ACC and HMG-CoA reductase the phosphorylation state and activity of these substrates show a diurnal rhythm (Davies SP *et al.*, 1992), whether AMPK activity shows diurnal changes is unclear.

The phosphorylation state of AMPK substrates can be altered in response to hormones. The diurnal rhythm of fatty acid and cholesterol synthesis due to alterations in ACC and HMG-CoA reductase phosphorylation reflect changes in the levels of insulin and glucagon, released during feeding and fasting respectively. Increasing insulin concentrations have been shown to be responsible for the dephosphorylation of HMG-CoA reductase (Easom RA and Zammit VA, 1987). Insulin and glucagon have been shown to regulate the phosphorylation state of ACC in rat liver *in vivo* (Mabrouk GM *et al.*, 1990) and in isolated rat hepatocytes (Sim ATR and Hardie DG, 1988).

Whether these phosphorylation changes are due to alterations in the activity of AMPK or protein phosphatases is not clear. Insulin treatment of hepatoma cells is associated with a small decrease in AMPK activity, with maximal inhibition occurring at insulin concentrations between 10 and 100nM (Witters LA and Kemp BE, 1992). An activation of liver AMPK has been measured in response to starvation, an effect which was inversely correlated with plasma insulin levels (Munday MR *et al.*, 1991). Physiological concentrations of insulin have been shown to inhibit AMPK in skeletal muscle *in vitro* and *in vivo*, and insulin failed to increase malonyl-CoA and decrease palmitate oxidation when AMPK was activated by incubation with AICAR (Winder WW and Holmes EW, 2000). In hearts perfused with 0.4mM palmitate raising the insulin concentration from 100μU/ml to 1000μU/ml resulted in an inactivation of AMPK and increased ACC activity (Gamble J and Lopaschuk GD, 1997). The activation of AMPK seen in rat hearts during ischaemia can be overcome by pre-treating hearts with insulin (Beauloye C *et al.*, 2001). In contrast with

these results Davies *et al*, 1992 observed no change in liver AMPK activity between light and dark periods and have suggested that the diurnal cycle of ACC and HMG-CoA reductase phosphorylation is due to alterations in protein phosphatase activity. In rat skeletal muscle incubated with pyruvate, insulin treatment (1μM) did not alter either α-1 or α-2 AMPK activity (Hayashi T *et al.*, 2000). No change in AMPK activity was seen when rat hearts were perfused with 0.4mM palmitate plus insulin 100μU/ml compared to controls perfused minus the hormone (Sakamoto J *et al.*, 2000). No effect of adrenaline on AMPK activity has been observed (Davies SP *et al.*, 1989).

HSL was later identified as a target of AMPK. Phosphorylation of ser-565 by AMPK prevents the subsequent phosphorylation and activation of the lipase by PKA at ser-563 and hence the adrenergically induced stimulation of TAG breakdown (Garton A.J. *et al.*, 1989). Inhibition of HSL by AMPK has been proposed to prevent the energy consuming recycling of free fatty acids into cholesterol esters or TAG when ATP levels are depleted (Hardie DG and Carling D, 1997).

The use of AICAR, the cell permeable activator of the AMPK signalling system, has allowed the effects of the kinase cascade to be studied in intact cells. Once inside cells AICAR is phosphorylated by adenosine kinase to its monophosphorylated form AICA riboside monophosphate or ZMP, which is an AMP analogue. ZMP is able to activate AMPK *in vitro* by direct allosteric effects (Sullivan JE *et al.*, 1994) and by promoting phosphorylation by AMPKK (Corton JM *et al.*, 1995). ZMP is not a specific activator of AMPK and it can also mimic the effects of AMP on other enzymes such as glycogen phosphorylase and fructose 1,6-bisphosphatase. However this method has proved useful in identifying *in vivo* substrates of AMPK.

Inhibition of fatty acid synthesis and cAMP-stimulated lipolysis occurs in response to AICAR induced activation of AMPK in adipocytes (Sullivan JE *et al.*, 1994). AICAR treatment of hepatocytes inhibits fatty acid and cholesterol synthesis (Corton JM *et al.*, 1995). AICAR treatment of hepatocytes also results in increased rates of fatty acid oxidation and CPT1 activity (Velasco G *et al.*, 1997). These changes could be largely attributed to decreases in malonyl-CoA concentration resulting from inhibition of ACC. AMPK inhibition

of liver ACC therefore plays a duel role in conserving ATP by simultaneously stimulating fatty acid oxidation and inhibiting fatty acid synthesis.

The AMPK induced inhibition of ACC appears to play a crucial role in exercising muscle when ATP becomes depleted. The decrease in malonyl-CoA concentration following ACC phosphorylation results in an increase in CPT1 activity, fatty acid oxidation and ATP generation. Reduced levels of malonyl-CoA, ACC activity and a stimulation of AMPK activity have been measured in the skeletal muscle of exercising rats (Winder WW and Hardie DG, 1996). In a later study using electrically stimulated skeletal muscle the same effects were observed and shown to correlate with an increase in the AMP/ATP ratio (Hutber CA *et al.*, 1997). In this study however, the inhibition of ACC preceded activation of AMPK and changes in AMP: ATP. Vavvas *et al*, 1997 showed that the activation of AMPK in electrically stimulated muscle was restricted to the α-2 isoform, and that the effect of contraction to inhibit ACC activity could be reversed by treatment with protein phosphatases. Large increases in fatty acid oxidation rates have also been measured in rat muscle in which AMPK had been stimulated by incubation with AICAR and these changes were associated with decreases in both ACC activity and malonyl CoA (Merrill GF *et al.*, 1997). This study also made the unexpected finding that AICAR –treated muscles also show a two-fold increase in glucose uptake, implying a role for AMPK in this effect. These observations have led to the proposal that during contraction the activation of AMPK is able to stimulate ATP generation both from enhanced fatty acid oxidation and increased glucose availability. The effects of AICAR treatment and contraction are not additive, and are not affected by the phosphotidylinositol 3-kinase inhibitor wortmannin (Hayashi T *et al.*, 1998). AMPK stimulation of glucose transport has been shown to involve increased rates of GLUT4 translocation in both heart (Russell AR *et al.*, 1999) and skeletal muscle (Kurth-Kraczek EJ *et al.*, 1999) by a mechanism distinct from that stimulated by insulin. A recent study, however, has suggested that the AMPK cascade may interact with the early events in insulin signalling (Jakobsen SN *et al.*, 2001). This was due to the observation that AICAR treatment of mouse myotubules and rat adipocytes and hepatocytes causes phosphorylation of the insulin receptor substrate 1 (IRS-1) and a stimulation of PI 3-kinase.

Heart tissue contains an active AMPK with the α-2 catalytic subunit being more abundant than α-1 (Stapleton D. *et al.*, 1996). Like skeletal muscle, the major role for cardiac AMPK appears to be the control of fatty acid oxidation rates via regulation of ACC activity. Purified rat heart ACC2 is phosphorylated and inactivated by incubation with purified rat liver AMPK (Dyck JRB *et al.*, 1999). In this study the α-2 isoform of AMPK was found to co-purify with ACC implying a role for this isoform in the regulation of heart ACC. This is in contrast to the situation in liver where the α-1 subunit has been predicted to regulate ACC activity (Michell B.J. *et al.*, 1996).

The majority of studies on cardiac AMPK have focused on alterations in kinase activity due to ischaemia and reperfusion when AMP levels would be expected to increase. Under normoxic conditions rates of fatty acid oxidation are linked to cardiac work with supply of acetyl-CoA from β-oxidation matched to meet the demands of the citric acid cycle. During reperfusion of ischaemic hearts there is an uncoupling between fatty acid oxidation and cardiac work and dramatic increases in rates of palmitate oxidation have been measured under these conditions (Kudo N *et al.*, 1995). An over-reliance on fatty acid oxidation is detrimental to the functional recovery of ischaemic hearts with hearts perfused in the absence of exogenous fatty acids having higher recovery rates (Saddik M and Lopaschuk GD, 1992). This effect has been proposed to be due to the fatty acid induced inhibition of PDH and hence glucose oxidation. Stimulation of glucose oxidation by activation of PDH or inhibition of CPT1 has been shown to improve the recovery of ischaemic hearts (McVeigh JJ and Lopaschuk GD, 1990).

Cardiac malonyl-CoA levels fall to 38% of aerobic control values during a period of global ischaemia, and decrease further (to 1% of controls) during a period of aerobic reperfusion (Kudo N *et al.*, 1995). These changes in malonyl-CoA correlate with increases in AMPK activity which is elevated during ischaemia. AMPK activity remained high during the reperfusion period despite AMP levels having returned to control values. Decreases in ACC activity in this study were not observed until the end of the aerobic reperfusion. The heart isoform of PFK2 has recently been identified as a substrate of AMPK (Marsin AS *et al.*, 2000). Activation of this enzyme by AMPK phosphorylation occurs during ischaemia and together with increased

glucose transport and is likely to be involved in the stimulation of glycolysis observed under anaerobic conditions.

The localisation of some of the AMPK α-2 isoform to the nucleus in INS-1 cells (Salt I.P. *et al.*, 1998) and the observation that the transcriptional coactivator p300 is a substrate for the kinase (Yang W *et al.*, 2001) gives support to suggestions that the AMPK system plays a role in transcriptional control. The AMPK is the mammalian equivalent of the yeast SNF1 protein which is a protein kinase essential for the transcription of glucose-repressed genes, and which allows yeast to grow on non-glucose sugars. In mammals glucose is able to up-regulate the transcription of a number of genes involved in carbohydrate and lipid metabolism in liver and adipose tissue.

The question as to whether the mammalian AMPK system is able to regulate glucose –dependent transcription has also been investigated. In cultured hepatocytes incubation with AICAR inhibited the glucose-dependent transcription of fatty acid synthase and the liver isoform of pyruvate kinase (Leclerc I *et al.*, 1998) (Foretz M *et al.*, 1998). In the study by Foretz *et al* the effect of glucose was indirect with no alteration in AMPK activity observed on increasing the glucose concentration from 5 to 25mM. Increasing the incubation glucose concentration from 3 to 30mM has been shown to inhibit AMPK activity in islet β cells (da Silva Xavier G *et al.*, 2000). In these cells AICAR also prevented the glucose-induced transcription of the L-pyruvate kinase gene. AICAR treatment of hepatocytes has also been shown to overcome the inhibitory effect of glucose on expression of the gluconeogenic phosphoenolpyruvate carboxykinase (PEPCK) gene (Hubert A *et al.*, 2000). Insulin suppresses the expression of PEPCK and gluconeogenesis in healthy liver. In contrast with the study of Hubert *et al* AICAR has been shown to mimic the effects of insulin to decrease PEPCK expression in hepatoma cells (Lockhead PA *et al.*, 2000).

1.7. Pathological Alterations in Heart Metabolism.

Although fatty acid oxidation can provide 60-70% of the energy requirements of the heart, certain pathological conditions can alter the relative contributions of fatty acid and carbohydrates to energy production. The reasons for the

change in fuel selected can include elevation of circulating fatty acids and inhibition of carbohydrate metabolism, carnitine deficiency and in some cases alterations in the enzymes of β-oxidation. This section aims to outline two conditions where alterations in cardiac metabolism occur, cardiac hypertrophy and diabetes.

1.7.1. Cardiac Hypertrophy.

The development of hypertrophy by the heart is associated with congestive heart failure, increased risk of myocardial infarction and sudden death. The hypertrophied heart also undergoes metabolic alterations and impaired energy metabolism has been proposed to play a role in the deterioration of heart function. Decreases in total tissue creatine (Conway MA *et al.*, 1991) and NADH (El Alaoui-Talibi Z *et al.*, 1997) levels have been measured in hypertrophied hearts implying an alteration in the intracellular energy reserve. Citrate synthase activity is reduced in hypertrophy and has been proposed to be responsible for the reduced oxidative capacity of affected hearts (Kalsi KK *et al.*, 1999).

Several structural changes are associated with cardiac hypertrophy. Myocytes increase in size with no increase in capillary density, thus delivery of oxygen and substrate to the hypertrophied heart is modified. The condition is also associated with a switch in cardiac fuel selection. Hypertrophied hearts show a decreased reliance on long chain fatty acid oxidation which provides only 55% of the hearts ATP requirements (El Alaoui-Talibi Z *et al.*, 1997). Whether this decrease in fatty acid oxidation is due to a re-routing of fatty acids to phospholipid synthesis is not clear. Unpublished results from this laboratory have shown that the activity of type 2 phosphatidate phosphohydrolase is increased in rats 4-6 weeks after aortic banding and returned to control values after 10 weeks. Fatty acyl-CoA synthetase activity was decreased and citrate –independent ACC activity increased in 10 week hypertrophied rat hearts, results that imply reduced flux through CPT1 by decreased substrate availability and increased malonyl-CoA.

Hypertrophied hearts show increased glycolytic flux with an uncoupling between glycolysis and glucose oxidation (Allard MF *et al.*, 1994). This uncoupling can be accounted for by the decreased amount of PDH in the active form in hypertrophied versus control hearts (Seymour A-ML and

Chatham JC, 1997). The reduced reliance on fatty acids by hypertrophied hearts has been proposed to be due to the decreased carnitine content of the tissue (Reibel DK *et al.*, 1994). Carnitine deficiencies result in reduced rates of fatty acyl-CoA translocation across the mitochondrial membrane and decreased substrate for β-oxidation. The decreased level of carnitine has also been suggested to modulate the activity of PDH by increasing the mitochondrial acetyl-CoA:CoASH. Hypertrophied hearts are able to oxidise short chain fatty acids suggesting that the enzymes of β-oxidation are unaltered and the oxidation of fatty acids is inhibited at the level of mitochondrial translocation.

Myocardial hypertrophy is also associated with the re-expression of the 'foetal' isoforms of certain proteins including β-myosin heavy chain and creatine kinase. Hypertrophied hearts also show decreased levels of GLUT4 and M-CPT1 mRNA, with no alteration in the mRNA of the 'foetal' GLUT1 and L-CPT1 (Depre C *et al.*, 1998). Unpublished results from this laboratory have obtained malonyl-CoA inhibition curves from hypertrophied hearts that also indicate an increased contribution from L-CPT1 in this tissue. L-CPT1 has a higher affinity for carnitine and is less sensitive to malonyl-CoA inhibition than the muscle isoform, and may serve to maintain low levels of fatty acid translocation and oxidation in hypertrophied heart tissue.

1.7.2. Diabetes.

In uncontrolled diabetes, despite a decreased carnitine content, the heart shows an increased dependence on fatty acid oxidation which is able to supply over 90% of the ATP requirements of the tissue (Wall SR and Lopaschuk GD, 1989). High rates of fatty acid oxidation are due to the high plasma concentration of fatty acids in diabetics as well as decreased rates of glucose uptake from the circulation, and an increase in the Km for glucose phosphorylation (Morgan HE *et al.*, 1961a). The enhanced rates of fatty acid oxidation observed in diabetic hearts has been proposed to involve the regulation of CPT1 by malonyl-CoA. No change in the sensitivity of CPT1 to malonyl-CoA occurs in diabetic heart tissue, and the de-inhibition of the enzyme is associated with decreases in the malonyl-CoA concentration of diabetic pig heart (Hall JL *et al.*, 1996). This decrease in malonyl-CoA concentration is not associated with changes in the activity of either ACC or

AMPK and has been proposed to result from the increased expression and activity of MCD measured in diabetic rat hearts (Sakamoto J *et al.*, 2000).

Increased rates of β-oxidation are associated with increased production of both acetyl-CoA and NADH, which are able to inhibit PDH. Glucose oxidation rates have been shown to be depressed in perfused diabetic hearts compared to controls (Saddik M and Lopaschuk GD, 1994). In this study no significant change in glycolytic rates due to diabetes was observed.

Diabetic hearts also have large stores of TAG compared to healthy tissue, and this is probably due to an increased fatty acyl-CoA content which results from increased fatty acid availability and the increased synthesis of CoASH observed in diabetic hearts (Lopaschuk GD *et al.*, 1986). This expanded TAG pool can be rapidly mobilised and is able to provide 70% of the ATP requirements of diabetic hearts perfused in the absence of exogenous fatty acid (Saddik M and Lopaschuk GD, 1994).

1.8. Aims of the Project.

The provision of long-chain fatty acids inhibits glucose utilisation in cardiac tissue. Oxidation of fatty acid is essential for this effect as it is abolished during ischaemia and by inhibitors of CPT1. Malonyl-CoA levels are increased in the heart by glucose, resulting in an inhibition of CPT1 and therefore rates of β-oxidation. A mechanism is therefore necessary by which fatty acids are able to stimulate a decrease in tissue malonyl-CoA prior to β-oxidation and preceding their inhibition of glucose metabolism.

The aim of this project was to test the hypothesis that fatty acids are able to 'feed-forward' their availability via an inhibition of ACC by the AMPK cascade. Prior to the CPT1 reaction fatty acids are first activated to their CoA derivatives. This activation is catalysed by fatty acyl-CoA synthetase and generates AMP in the reaction: fatty acid + ATP + CoASH ↔ acyl-CoA + AMP + PPi. This AMP could potentially stimulate the upstream AMPKK and increase the activity of AMPK directly as well as by increased protein phosphorylation.

Alterations in rates of fatty acid oxidation (via changes in malonyl-CoA) occur in the heart in response to hormones and in certain pathological conditions. Therefore an additional aim of the project was to test the relationship between malonyl-CoA concentration and the activities of both ACC and AMPK in hearts perfused with insulin or adrenaline, and in hearts removed from rats in which cardiac hypertrophy was induced by aortic banding.

Chapter Two

Materials and Methods.

2.1. Commercial Preparations.

All routinely used chemicals were obtained from Sigma Chemical Co. Ltd, Poole, Dorset U.K. The following were supplied by:

Sigma Chemical Co. Ltd.: Adenosine 5'- triphosphate (disodium salt), Protein G-sepharose, Bovine serum albumin (essentially fatty acid free, fraction V), Palmitic acid (sodium), Malonyl CoA (lithium), Acetyl CoA (sodium), Adenosine 5'-diphosphate (disodium), Adenosine 5'-monophosphate (disodium), L-epinephrine (hydrochloride), Insulin and Bicinchoninic acid protein assay kit.

Roche : NADH (reduced form, sodium salt), NADPH (reduced form, sodium salt), Lactate Dehydrogenase (from pig heart), Pyruvate kinase, Glycerol kinase and Phosphoenolpyruvate.

Amersham International : Sodium [^{14}C] bicarbonate, [^{3}H] Acetyl-CoA.

N.E.N. : γ- [^{33}P]- ATP.

BOC Ltd., Pressurised gasses, O_2:CO_2 95%: 5%, O_2–free N_2, and liquid N_2.

Alpha Laboratories Ltd.: Wako NEFA C Test kit.

SAMS peptide was obtained from Dr. M. Munday, University of London School of Pharmacy.

AMPK antisera and **Phosphoprotein Phosphatase 2C** were gifts from Dr. D. Carling, MRC Clinical Sciences Centre, Imperial College School of Medicine, Hammersmith Hospital, London.

2.2. Laboratory Preparations.

2.2.1. Palmitate Bound To Albumin.

The preparation of palmitate bound to albumin was carried out as described previously (Evans and Muller, 1963). 250mg of sodium palmitate was added per 50ml of a solution of 15% (w/v) of bovine serum albumin (BSA) in 0.9% (w/v) NaCl. After mixing the solution was sonicated in a sonicating water bath until an even mixture was obtained. The resulting suspension was incubated at 50°C for 30 minutes before being left at 4°C overnight. The solution was then filtered at 4°C through Whatman No.1 filter paper to remove microcrystals of sodium palmitate, followed by centrifugation at 26,000g_{av} for 30 minutes at 4°C in a Beckman Sorvall RC5-B centrifuge. The supernatant was collected and the pH adjusted to 7.4 with NaOH and stored in aliquots at -20°C. The concentration of bound palmitate was determined using the Wako C NEFA kit (Section 2.6.4.).

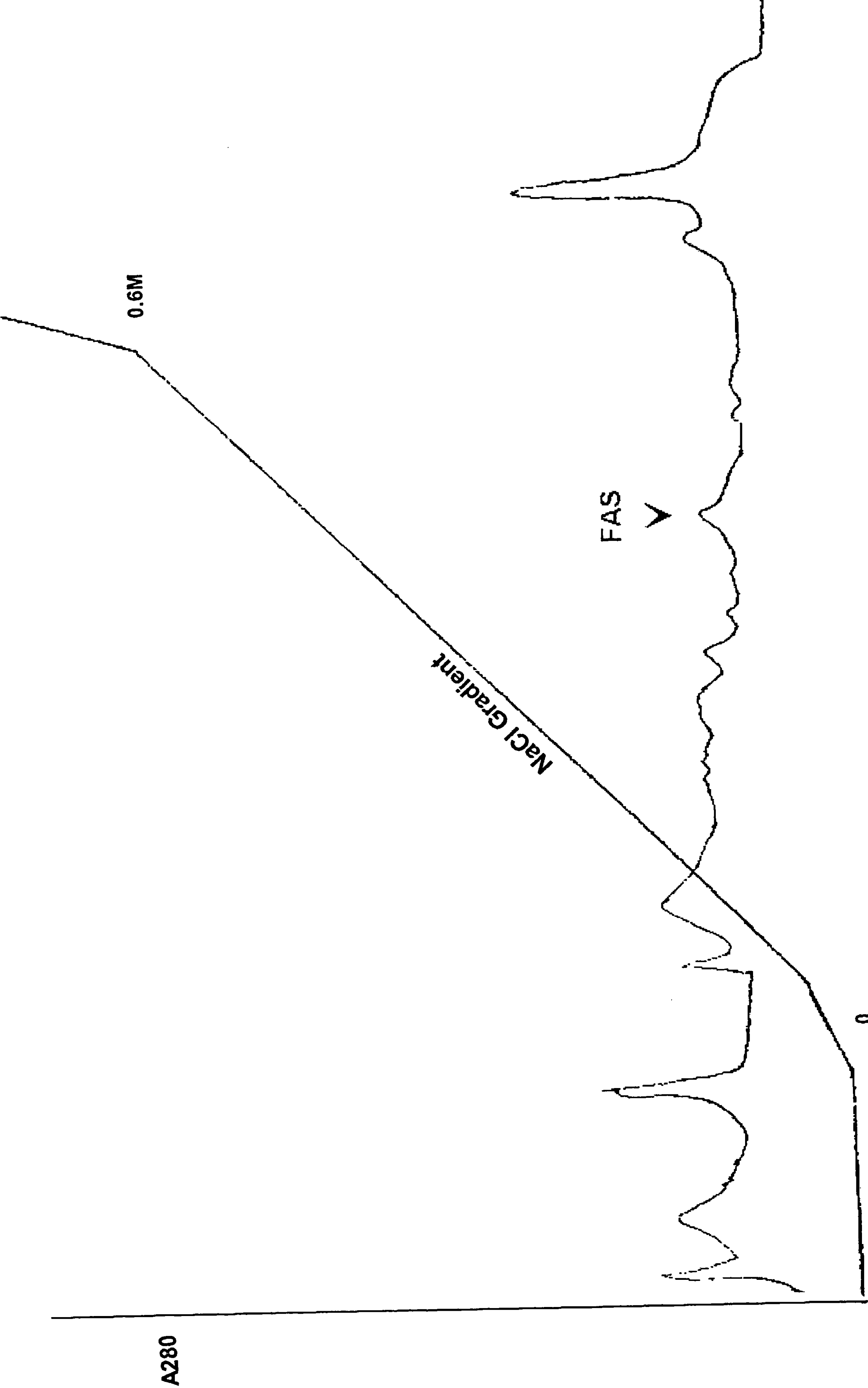
2.2.2. Preparation of AMPK Antibodies/Protein G.

Protein G sepharose was washed and resuspended as a 50:50 slurry in a 50mM Hepes buffer pH 7.4 containing 50mM NaF, 5mM $Na_4P_2O_7$, 10% (v/v) glycerol, 1mM EDTA and 1mM dithiothrietol (DTT). 100µl of either anti- α1 or α2 AMPK antiserum, or pre-immune sheep serum as controls, was added to 500µl of slurry and mixed at 4°C for 2 hours. After binding the slurries were washed twice in Hepes buffer as above and stored at 4°C until used.

2.2.3. Purification of Fatty Acid Synthase.

Fatty acid synthase (FAS) was purified from rat liver by a method similar to that of Hsu *et al,* (1965). Frozen livers were weighed, washed and chopped in ice-cold homogenisation medium (5ml per gram of liver) containing 10mM Tris-HCl (pH 7.4), 225mM mannitol, 75mM sucrose, 0.5mM EDTA and 0.5mM DTT. The tissue was then homogenised on ice by 3-4 strokes of a motor driven teflon pestle (450 rev/min) in a glass Potter Elvehjem homogeniser (radial clearance 0.19mm).

Figure 2.1. FPLC Elution of FAS.



The resulting homogenate was centrifuged at 9,000g_{av} for 10 minutes at 0-4°C in a Beckman Sorvall RC5-B centrifuge. The resulting supernatant was centrifuged at 100,000g_{av} in a Beckman L8 ultracentrifuge for 1 hour at 0-4°C. The supernatant was taken and made up to 25% saturation by the slow addition of ice cold, saturated ammonium sulphate solution, and stirred at 4°C for 30 minutes. The mixture was then centrifuged at 18,000g_{av} for 10 minutes and the pellet discarded. The supernatant was brought up to 40% saturation by addition of more saturated ammonium sulphate, mixed and centrifuged as above. The resulting precipitate was resuspended in a minimum volume of 20mM bis-Tris propane buffer pH 7.0, containing 30mM NaCl, 3mM EDTA and 1mM DTT and desalted by overnight dialysis in 100x volume of the same buffer. The dialysed solution was applied to a Pharmacia FPLC Hiload 16/10 Q Sepharose column previously equilibrated with 20mM bis-Tris propane buffer (pH 7.0) containing 30mM NaCl, 3mM EDTA and 1mM DTT. The enzyme was eluted using a linear gradient of 0-0.6M NaCl with two column volumes (flow rate 2ml/min) of the same buffer (Figure 2.1.). FAS activity was eluted between 0.2 and 0.25M NaCl and its activity determined (section 2.8.3.). The enzyme was stored in aliquots as a 10% glycerol solution at -70°C until required.

2.3. Animals.

Animals used were male Sprague Dawley rats (300-350g) bred in the Biological Services Unit at UCL.

Rats were housed at 20-22°C with constant access to drinking water and to Rat and Mouse Breeding Diet (Special Diet Services, Witham, Essex, UK). The diet contained 21% protein, 4% fat and 39% starches and sugars. The light/dark cycle was 13 hours of light between 0600 and 1900h.

2.4. Heart Perfusion.

Animals were anaesthetised by an intraperitoneal injection of sodium pentobarbitone (300mg/Kg). Hearts were excised and arrested by placing them in ice cold Krebs Henseleit Bicarbonate buffer (KHB-made up of 25mM $NaHCO_3$, 118mM NaCl, 4.7mM KCl, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4) pH7.4 containing 10mM glucose and 2.5mM $CaCl_2$. Hearts were then cannulated by the aorta to the perfusion apparatus whilst visible fat was trimmed.

Langendorff perfusions were carried out for 1 hour at 37°C with KHB (pH 7.4) containing 5mM glucose, 1.3mM $CaCl_2$ and 2%w/v BSA, against 80 ± 5cm of hydrostatic pressure, as previously described (Mowbray J and Ottaway JH, 1973). The perfusion medium was gassed constantly with O_2:CO_2 (95%:5%). The majority of perfusions were carried out with a re-circulating perfusion medium. Where adrenaline was included the perfusate was collected after one passage through the heart. Additions to the perfusion media of palmitate (bound to albumin), adrenaline, insulin and pyruvate were at concentrations referred to in the text. After 60 minutes of perfusion hearts were excised from the cannula and quickly frozen between tongs pre-cooled to the temperature of liquid N_2. Heart tissue was then powdered under liquid N_2 and stored at -70°C. Samples of perfusion media were collected and frozen at -70°C for determination of NEFA (Section 2.6.4.).

2.4.1. Preparation of Neutralised Acid Extracts.

5ml samples of perfusion medium were acidified by addition of 400μl of perchloric acid 60%. The extracts were centrifuged at 2000g_{av} for 5 minutes to remove precipitated protein. 400μl of triethanolamine 1M was added to the resulting supernatants and the sample pH adjusted to 7.0 by the addition of saturated K_2CO_3. The samples were then left on ice for 5 minutes and respun as above to remove precipitated potassium perchlorate. The supernatants were removed and stored at -70°C, prior to assay of glycerol content (section 2.6.2.).

2.5. Tissue Preparation for Metabolite Measurements.

2.5.1. Trichloroacetic Acid Extraction.

Powdered frozen heart tissue was weighed and extracted by addition of TCA: methanol (10%: 25%) in 10mM $MgCl_2$, 1ml per 50mg of tissue. The extract was mixed and centrifuged at 26,000g_{av} for 10 min at 4°C. The supernatant was washed with 3x 5ml of H_2O saturated diethylether then gassed for 1 h with O_2 - free N_2. The pH of the samples was then adjusted to pH7.0 by addition of crystals of Tris base. Samples were then immediately assayed for adenine nucleotide content (section 2.6.1).

2.5.2. Perchloric Acid Extraction.

Powdered heart tissue was weighed and extracted by the addition of 6% (v/v) PCA (200mg/ml). Samples were then homogenised on ice using an Ultra Turrax tissue disintegrator and centrifuged at $2000g_{av}$ for 10 minutes at 4°C. The supernatant was removed and 1M triethanolamine added (0.5ml per ml of supernatant). The pH of the sample was then adjusted to 7.0 using KOH (10M then 1M). Samples were then left for 30 minutes on ice and respun as above. The resulting supernatants were collected and appropriate volumes used immediately for the determination of malonyl-CoA content (section 2.6.3.). The weight of tissue used and the volumes of all additions were recorded.

2.6. Metabolite Determination.

2.6.1. Adenine Nucleotides.

Tissue adenine nucleotide content was determined by HPLC analysis as described previously (Merrill GF *et al.*, 1997). Samples for the analysis of adenine nucleotides were prepared as described in section 2.5.1. and filtered through a 0.22μm millipore filter. HPLC analysis was then carried out by injection of 100μl of sample onto a 150 x 4.6mm Primesphere NH_2 column (5 micron pore size). The column was pre-equilibrated at room temperature with 5mM KH_2PO_4, pH 3.0 at a flow rate of 1ml/min (1000 Bar pump pressure). Adenine nucleotides were eluted with a gradient rising to 500mM KH_2PO_4, pH 3.0 over 30 min. All buffers were filtered through a 0.22μm millipore filter and degassed before use. The eluted nucleotides were detected by the change in absorbance at 259nm which was monitored by a Waters 485 tunable absorbance detector. The detector response was recorded and processed using Millennium 2010 software. Standard solutions of ATP, ADP and AMP were included in each HPLC run. Sample peaks were identified by comparison of their retention times with standards or in the case of AMP by the inclusion of internal standards. The concentration of each nucleotide was determined from its peak area.

2.6.2. Glycerol.

Glycerol efflux into the perfusion media was determined spectrophotometrically using the method of Garland and Randle, 1962. Neutralised acid extracts prepared as described in section 2.4.1. were assayed in a final volume of 1ml containing 50mM triethanolamine pH 7.4, 5mM $MgCl_2$, 5mM KCl, 0.3mM ATP, 0.4mM phosphoenolpyruvate, 0.2mM NADH, 20μg pyruvate kinase and 20μg lactate dehydrogenase. The assays were carried out at 25°C and initiated by the addition of 10μg of glycerol kinase. The change in absorbance relative to a blank sample containing water instead of sample was recorded at 340nm and glycerol content calculated using the extinction coefficient of NADH (6.22$\mu mol^{-1}ml$).

2.6.3. Malonyl-CoA.

Malonyl -CoA was assayed by the method of McGarry *et al* (1978). Assays were carried out in duplicate in glass test tubes with a final volume of 1ml containing 0.2M KH_2PO_4 (pH 7.0) with 2mM EDTA, 0.1% (w/v) BSA, 2.5mM DTT, 0.25mM NADPH, 1.0μM [^{3}H] acetyl-CoA (180-250dpm/pmol) and 100μl of heart extract or malonyl-CoA standard. The reaction was initiated by the addition of 1mU of purified FAS (section 2.2.3). After a 90 minute incubation at 37°C the reactions were terminated by the addition of 29μl of 60% (w/w) perchloric acid. 1ml of ethanol was added to each tube followed by 5ml of petroleum ether (b.p. 60-80°C). The tubes were then vortex mixed and centrifuged at 2000rpm for 2minutes at room temperature in a Beckman RT7 centrifuge in order to improve the separation between the aqueous and organic phases. 4ml of the organic solvent layer was then transferred to a glass tube containing 2ml of distilled water, vortexed and re-centrifuged as above. 3ml of the resulting organic phase was again mixed with 2ml distilled water. After a final vortex mix and centrifugation, 2ml of the organic layer was placed in a glass scintillation vial and left in a fume cupboard overnight to evaporate to dryness. 10ml of Ecoscint A was added to each tube for scintillation counting. Sample concentrations of malonyl-CoA were determined by comparison with a standard curve of known malonyl-CoA concentrations (0-100 pmoles) which were included with every assay. Blank

Figure 2.2. Malonyl-CoA Standard Curve.

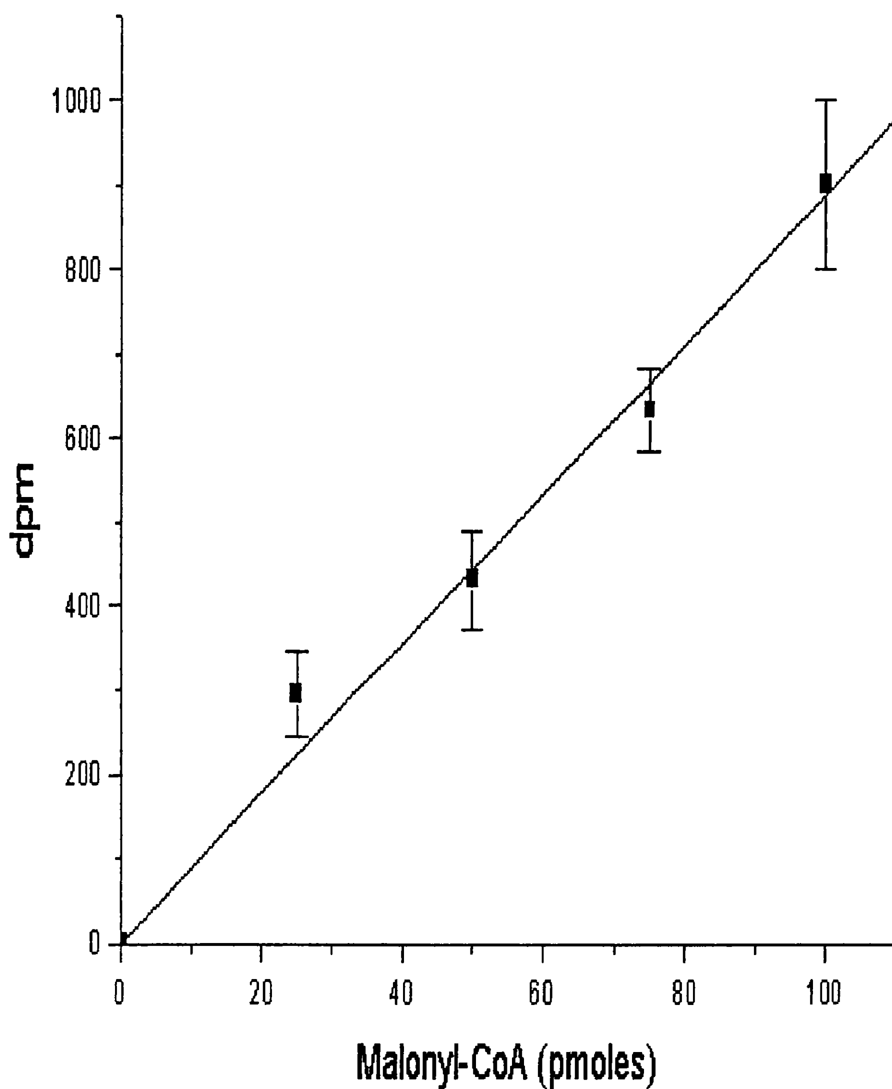


Figure 2.2. Standard curve showing average cpm for 0-100 pmoles of malonyl CoA. Error bars are SEM of 12 independent experiments.

assays that contained no tissue extract were also carried out with each set of samples .

2.6.4. Non-Esterified Fatty Acids.

Fatty acid concentrations were determined using the Wako NEFA C test kit (Mulder C *et al.*, 1983). Assays were carried out at 37°C in 96 well microtitre plates. 67μl of reagent A (50mM phosphate buffer pH 6.9 containing 3mM $MgCl_2$, 0.3U/ml acyl- Coenzyme A synthetase, 3U/ml ascorbate oxidase, 30mg/ml ATP and 7mg/ml Coenzyme A was incubated with 10μl of sample. This reaction catalyses the conversion of fatty acids to acyl CoA thioesters. After 10 minutes 133μl of reagent B (1.2mM 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA), 6.6U/ml acyl-Coenzyme A oxidase and 7.5U/ml peroxidase) was added and a further 10 minute incubation took place. In the second stage of the reaction the acyl-CoA thioesters are oxidised, producing hydrogen peroxide as a by-product. In the presence of peroxidase the hydrogen peroxide reacts with MEHA to form a purple coloured adduct. After cooling the absorbance was read on a Dynatech MR7000 spectrophotometer at 570nm against a blank incubation containing 2% BSA. NEFA concentrations were determined from standard oleate samples which were included in every assay.

2.7. Preparation of Tissue for Enzyme Assays.

2.7.1. Polyethylene Glycol Precipitation.

Polyethylene glycol (PEG) precipitation was carried out as previously described (Belke DD *et al.*, 1998). 200μl of frozen, powdered heart tissue was homogenised on ice in 0.4ml of buffer containing 50mM Tris/HCl (pH 7.8/25°C), 250mM mannitol, 1mM EDTA, 1mM EGTA, 50mM NaF, 5mM $Na_4P_2O_7$, 1mM PMSF, 1mM benzamidine, 1mM DTT and 4μg/ml soy bean trypsin inhibitor. The resulting homogenate was then centrifuged at 14,000g at 4°C for 20 minutes. The supernatant was removed and an appropriate volume of 25% PEG 6000 added to make a final concentration of 2.5%. The

sample was then vortex mixed and centrifuged at 10,000g for 10 minutes at 4°C. The resulting supernatant was then made up to a 6% PEG concentration by the addition of 25% stock solution (3.5μl per 19μl of supernatant). The sample was then vortex mixed and centrifuged as above. The final PEG pellet was then resuspended in 100mM Tris/HCl (pH 7.8/25°C) containing 1mM DTT to give a protein concentration of approximately 1mg/ml and used immediately for assay of AMPK activity (section 2.8.1).

2.7.2. Immunoprecipitation.

Powdered, frozen heart tissue was weighed and homogenised on ice (100mg/ml) using an Ultra Turrax tissue disintegrator in a 50mM Tris-HCl buffer (pH 7.8/ 25°C) containing 0.25M mannitol, 1mM EDTA, 1mM EGTA, 50mM NaF, 5mM $Na_4P_2O_7$, 1mM DTT, 1mM PMSF, 1mM benzamidine and 4μg/ml soybean trypsin inhibitor. The homogenate was then centrifuged at 13,000g_{av} for 10minutes at 4°C. AMPK immunoprecipitation was carried out as described previously (Woods A *et al.*, 1996). 250μl of supernatant was incubated for 2h at 4°C with 15μl of either α-1 or α-2 AMPK antibody pre-bound to protein G-sepharose (section 2.2.2.), or with 15μl of bound pre-immune sheep serum as controls.

At the end of the incubation the beads were pelleted by centrifuging at 5,200g_{av} for 1 minute. The beads were then washed with 300μl of homogenisation buffer and re-pelleted by centrifuging as above. The beads were washed a further two times in 300μl of AMPK assay buffer, 40mM Hepes pH7.0 containing 80mM NaCl, 0.8mM EDTA, 8% glycerol and 1mM DTT, with a one minute spin between each wash. After the final wash step the pellet was collected and any remaining liquid removed using a Hamilton syringe. AMPK activity was measured in the recovered pellets as described in section 2.8.1.

2.7.3. Phosphoprotein Phosphatase 2C Treatment.

For assays of AMPK after phosphatase treatment immunoprecipitates were prepared as above, with modifications to the washing procedure as described in Stein *et al* 2000. After the 2h incubation pelleted beads were washed once in homogenisation buffer followed by two washes in 300μl of a 50mM Tris-HCl

buffer pH 7.4, containing 1mM DTT in order to remove protein phosphatase inhibitors present in the immunoprecipitation buffer.

After the final wash pellets were collected, dried and incubated for 30min at 30°C in a final volume of 25μl containing 50mM Tris-HCl pH 7.4, 1mM DTT, 4μg PP2C (in 50% glycerol), and 10mM $MgCl_2$. Incubations were also carried out without added $MgCl_2$ as controls. At the end of the incubation period beads were collected by centrifugation at 5,200g_{av} for 1 min and washed 3 times in 300μl assay buffer (40mM Hepes pH7.0 containing 80mM NaCl, 0.8mM EDTA, 8% glycerol and 1mM DTT). After the final wash beads were collected by centrifuging as above and liquid removed using a Hamilton syringe. AMPK activity was then measured in the recovered pellets as described in section 2.8.1.

2.7.4. Preparation of 100,000g Supernatants.

Frozen, powdered heart tissue was weighed and homogenised (200mg/ml) on ice using an Ultra Turrax tissue disintegrator. The homogenisation buffer was 50mM Tris-HCl (pH 7.5/ 25°C), containing 250mM mannitol, 1mM EDTA, 1mM EGTA, 1mM DTT, 50mM NaF, 5mM $Na_4P_2O_7$, 1mM PMSF, 1mM benzamidine and 4μg/ml soybean trypsin inhibitor. The homogenate was centrifuged at 4°C for 10 min at 9,200g_{av} using a Beckman TL-100 ultracentrifuge. The supernatant was collected and centrifuged at 100,000g_{av} for 1h at 4°C. The 100,000g supernatant was collected and used immediately for ACC assays, the final membrane pellet was resuspended in 1ml of homogenisation buffer and also assayed.

2.8. Enzyme Assays.

2.8.1. AMP- Activated Protein Kinase.

Immunoprecipitates were prepared as described in section 2.7.3. and assayed for kinase activity in a final volume of 75μl of 40mM Hepes buffer pH 7.0 containing 80mM NaCl, 0.8mM EDTA, 8% glycerol, 1mM DTT, 5mM $MgCl_2$, 200μM SAMS peptide ± 200μM AMP (Davies SP *et al.*, 1989). Assays were carried out for 30 min at 37°C and initiated by the addition of 200μM γ-[^{33}P] ATP (250-500dpm/pmol). The reactions were stopped by placing 20μl of reaction mixture onto squares of Whatman P81

phosphocellulose paper which were immersed in 1% orthophosphoric acid. Papers were then washed twice for 10 min in 1% orthophosphoric acid, followed by two washes of 10 min in distilled H_2O. The papers were air-dried and placed into scintillation vials with 10ml Ecoscint A and the radioactivity counted. Activity was calculated after subtraction of values for control samples which were either immunoprecipitated with pre-immune sheep serum or assayed in the absence of SAMS peptide.

2.8.2. Acetyl-CoA Carboxylase.

Acetyl-CoA carboxylase activity was measured radiochemically by the method of Holland *et al*, 1986. 200μl of tissue extract (section 2.7.4.) was assayed in a final volume of 1ml containing 100mM Tris-HCl (pH 7.2), 2mM $MgCl_2$, 1.2mM DTT, 20mM $NaH^{14}CO_3$ (0.1μCi/μmol), 0.3mM acetyl-CoA, BSA 0.1% (w/v) and 4mM ATP. Assays were performed at 37°C for 6 minutes, plus and minus 10mM trisodium citrate which included an equimolar concentration of $MgCl_2$ to compensate for chelation of Mg^{++} by citrate. Reactions were terminated by the addition of 200μl of 6M HCl and samples placed on ice for 5 min. Blank reactions included in every assay were treated with HCl at zero time. The samples were centrifuged at $2000g_{av}$ for 5 min to remove precipitated protein.

0.8ml of the acid aqueous phase was placed into scintillation vials and evaporated to dryness to remove unreacted $^{14}CO_2$. 10ml of Ecoscint A was added to each vial for scintillation counting. ACC assays were linear with time for 6 minutes (figure 2.3.).

2.8.3. Fatty Acid Synthase.

Fatty acid synthase was measured spectrophotometrically using a method based on Saggerson and Greenbaum, 1970 . Assays were carried out in duplicate at 25°C in a 1ml cuvette containing 100mM KH_2PO_4 (pH 6.6) with 2mM EDTA, 2mM DTT, 0.25mM NADPH, 0.03mM acetyl-CoA and 100μl of purified FAS. The reaction was initiated by the addition of 0.05mM malonyl-CoA and the decrease in absorbance at 340nm measured

Figure 2.3. ACC Activity Time Course.

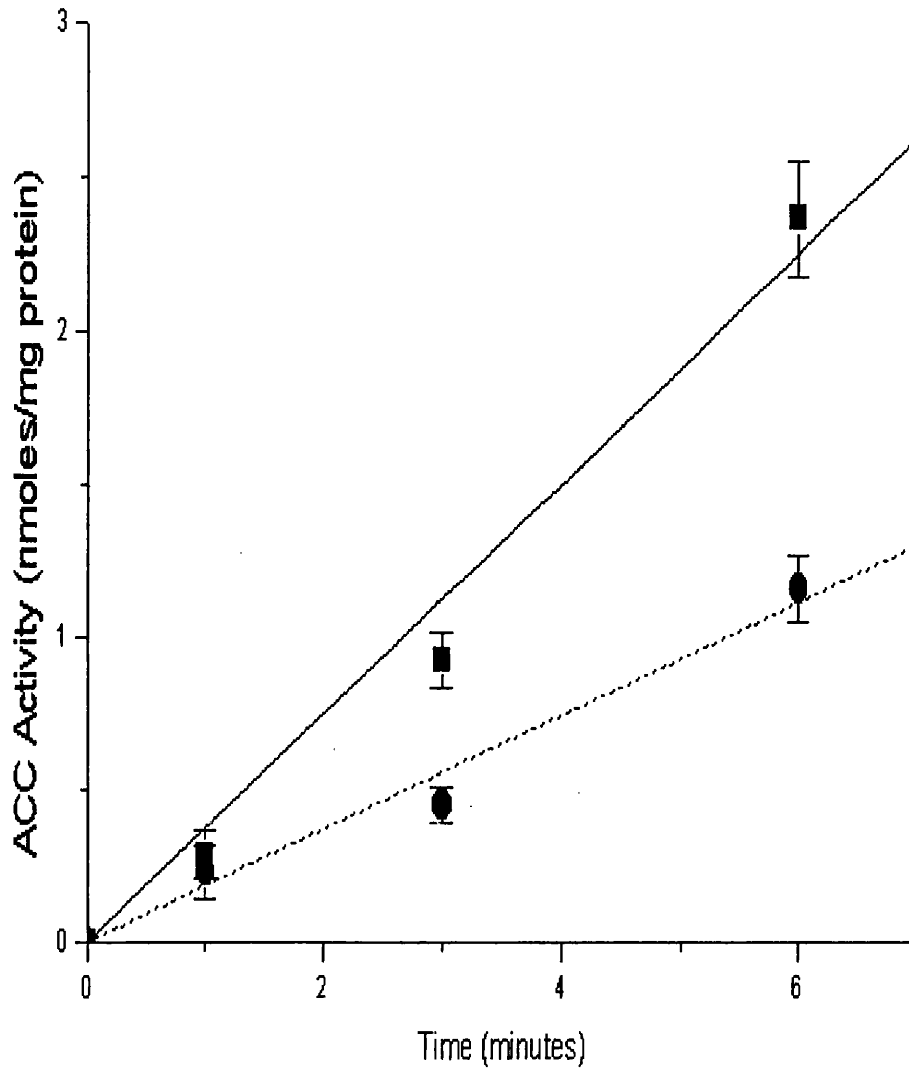


Figure 2.3. Heart tissue was extracted as described in section 2.7.4. and 100,000g supernatants assayed for ACC activity in the presence (solid line) or absence (broken line) of 10mM sodium citrate.

Values are the mean ± SEM for 5 independent assays.

against a blank reaction where malonyl-CoA was replaced by H_2O using a Unicam SP8-10 spectrophotometer. FAS activity was calculated using the extinction coefficient for NADPH of 6.22μmol^{-1} ml.

2.9. Protein Determination.

The protein concentration of tissue extracts was determined using a bicinchoninic acid (BCA) protein assay kit. The BCA reagent was prepared by adding 1 part copper (II) sulphate pentahydrate 4% solution to 50 parts bicinchoninic acid. 10μl of sample was incubated at 37°C with 200μl of the BCA/Cu^{++} mixture in a 96 well microtitre plate. After 30 minutes the plates were read against blank samples at 570nm using a Dynatech MR7000 spectrophotometer. The protein concentration in tissue samples was determined from BSA standard solutions (0.2-1.0mg/ml) which were included in each assay.

2.10. Scintillation Counting.

The radioactivity resulting from enzyme assays was measured on a Packard Tri-Carb 1900 analyser (Canberra Packard, Pangbourne, Berks.,U.K.) which is programmed for counting ^{33}P and ^{14}C isotopes. The counter is equipped with a computer which stores quench curves for various isotopes and allowed direct printing of activity in disintegrations per minute (dpm).

2.11.Statistical Methods.

Statistical significance between populations was determined using Students t-test for paired or unpaired samples as appropriate. Values are given as means plus and minus S.E.M. The n values quoted refer to the number of independent preparations.

Chapter Three

Results and Discussion.

3.1. Method Development.

AMPK activity was measured by following the incorporation of ^{33}P from γ-[^{33}P]-ATP into a synthetic peptide substrate, 'SAMS' peptide, which is based on the AMPK target sequence of rat liver ACC. The assay components and method (section 2.8.1.) was identical to that widely employed for the measurement of the kinase, except that ^{33}P rather than ^{32}P ATP was used in these experiments.

3.1.1. PEG Precipitation.

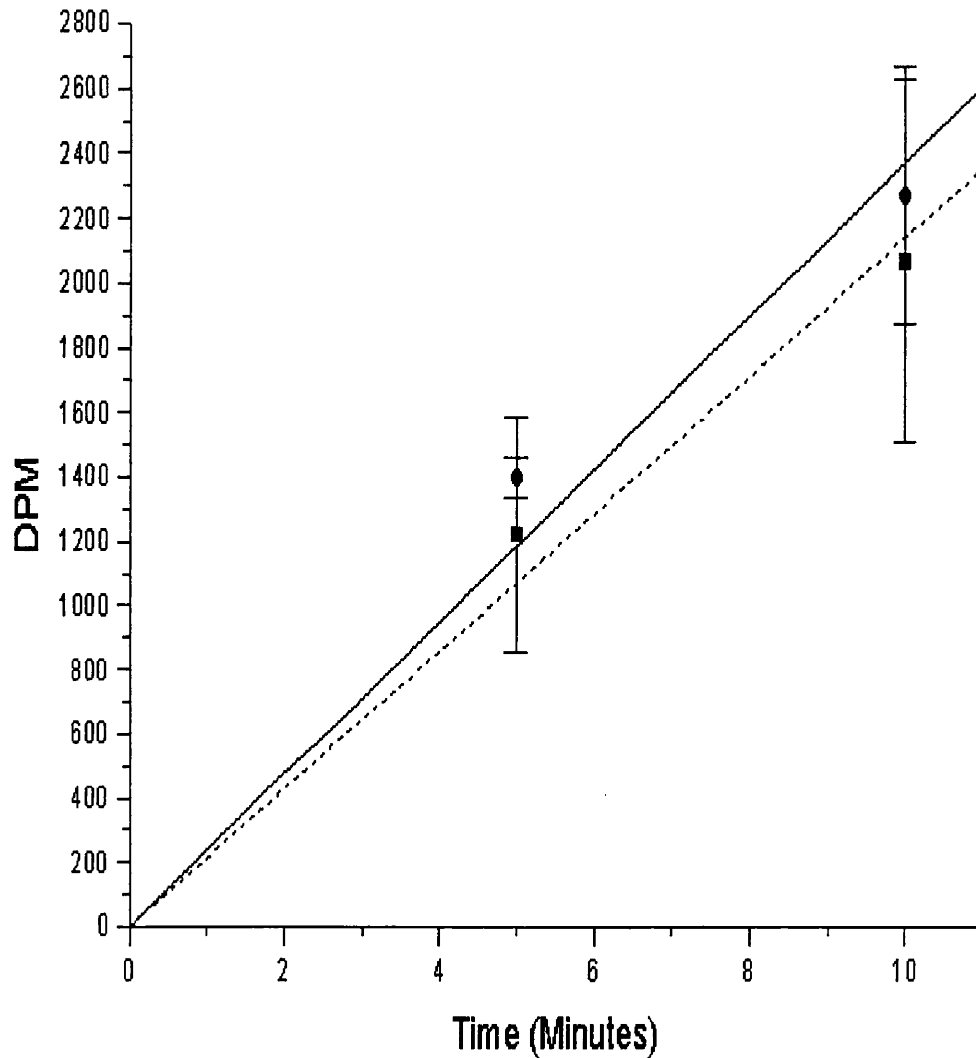
Initial measurements of AMPK activity in heart tissue were carried out using crude 13,000g supernatants of varying protein concentration. It quickly became clear that a more purified enzyme fraction was necessary to generate accurate and reproducible results and therefore the homogenised heart tissue was first subjected to polyethylene glycol precipitation as described in section 2.7.1.

AMPK activity was assayed in the 6% PEG pellet, resuspended to give a protein concentration of 1mg/ml as described by Belke *et al* (1998). Enzyme activity was linear with time for 10 minutes, see figure 3.1. However the inclusion of 200μM AMP into the assay buffer did not result in any stimulation of AMPK activity.

In order to determine whether the problem was with the AMPK assay itself or the tissue extraction method, assays were carried out using samples of DEAE purified liver AMPK obtained from Dr.M.Munday. As can be seen from figure 3.2. ^{33}P incorporation into SAMS peptide was linear for 10 minutes and a significant stimulation of activity in the presence of 200μM AMP was observed.

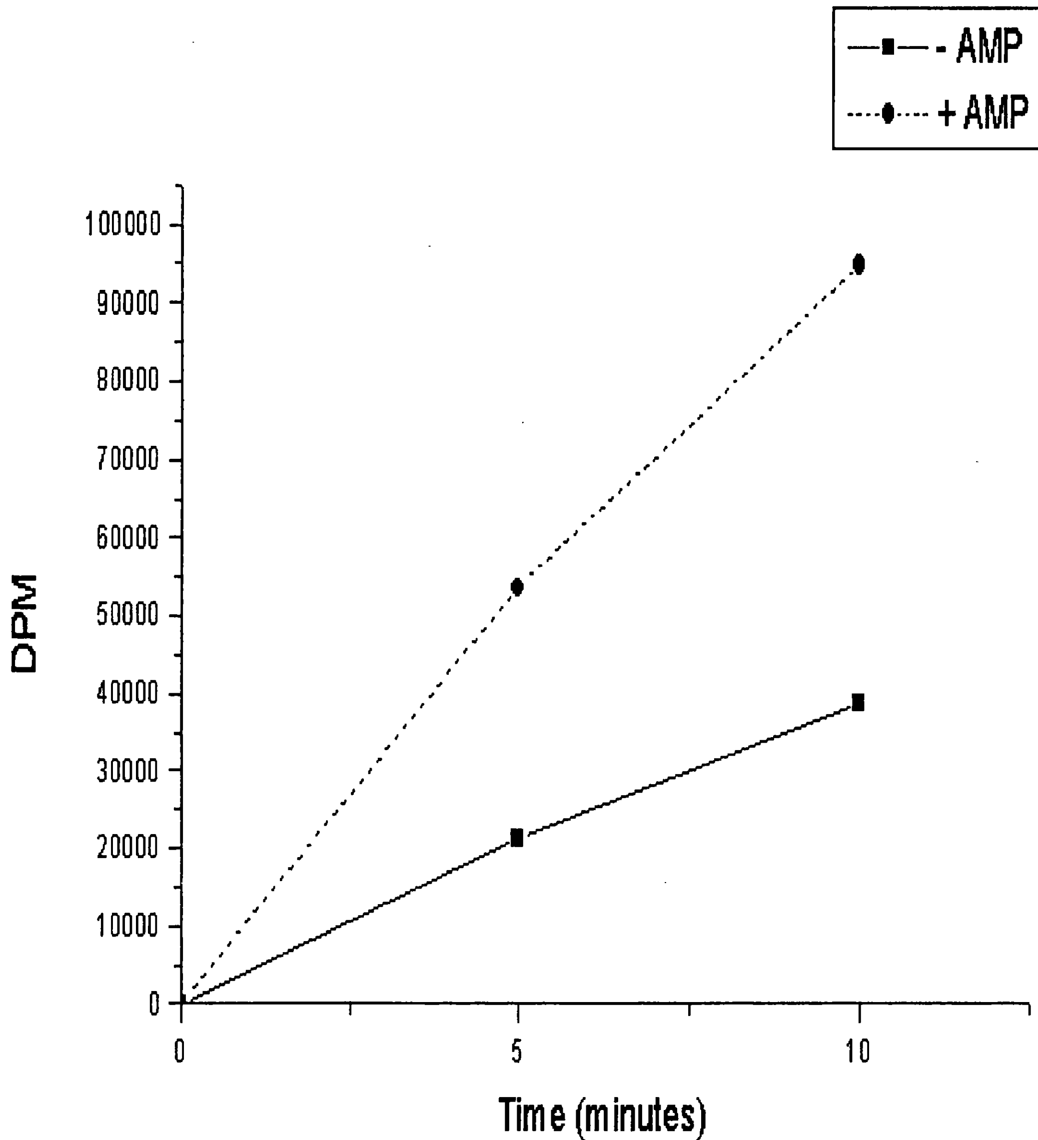
In a further set of experiments AMP was replaced in the assay by its analogue AICA riboside monophosphate (ZMP). The rationale behind this being the possibility that the PEG pellet might contain adenylate kinase activity which could effectively be removing AMP in the reaction AMP+ ATP$\leftrightarrow$ 2 ADP. Figure 3.3 shows that ZMP was also unable to stimulate AMPK activity in 6% PEG pellets.

Figure 3.1. AMPK Activity after PEG Precipitation.



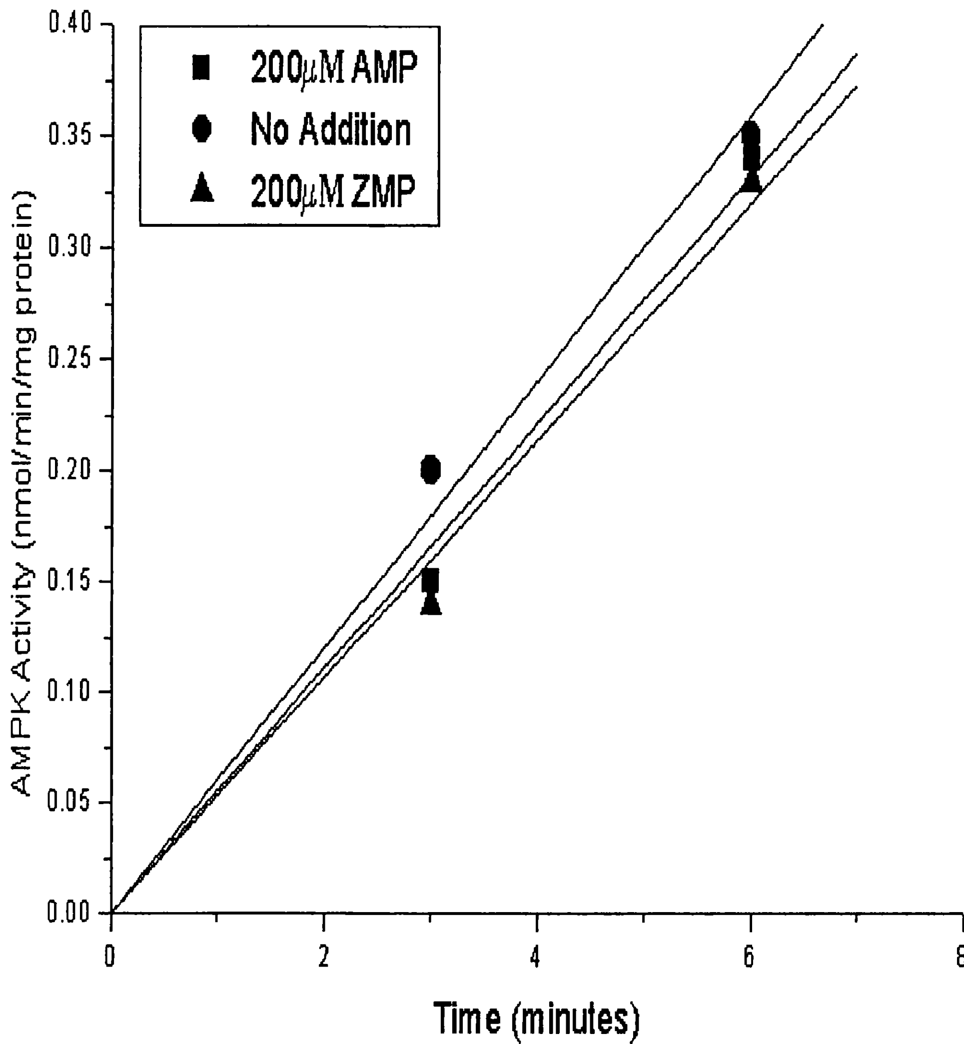
Hearts were perfused with 5mM glucose, 0.5mM palmitate and 2% BSA. AMPK activity was measured in the 6% PEG pellet plus (broken line) or minus (solid line) 200μM AMP after 5 and 10 minutes and expressed as the mean ± SEM for 4 independent assays.

Figure 3.2. AMPK Assay using DEAE Purified Liver Kinase.



To establish the efficiency of the AMPK assay method, the incorporation of ^{33}P into SAMS peptide was measured using purified rat liver AMPK (5μl) ± 200μM AMP. Figures shown are the disintegrations per minute for a single test assay.

Figure 3.3. Effect of ZMP on AMPK Activity in 6% PEG Pellets.



Hearts were perfused with 5mM glucose, 2% BSA and 0.5mM palmitate. Assays were carried out after PEG precipitation for 3 or 6 minutes with no addition or with either 200μM AMP or ZMP. The values shown are AMPK activity measurements of a single perfused heart.

The reason why no stimulation of AMPK activity by either AMP or ZMP could be observed in PEG pellets remains unclear. It is possible that the PEG pellet retains some endogenous cardiac AMP which masks the effect of AMP added to the assay buffer, effectively causing all measurements of AMPK to be AMP- stimulated. However this method of preparing heart samples for the assay of AMPK has been employed successfully by the Lopaschuk group who consistently demonstrate 2-3 fold stimulation of kinase activity assayed in 6% PEG pellets (Belke DD *et al.*, 1998; Lopaschuk GD *et al.*, 1994; Dyck JRB *et al.*, 1999).

3.1.2. Immunoprecipitation.

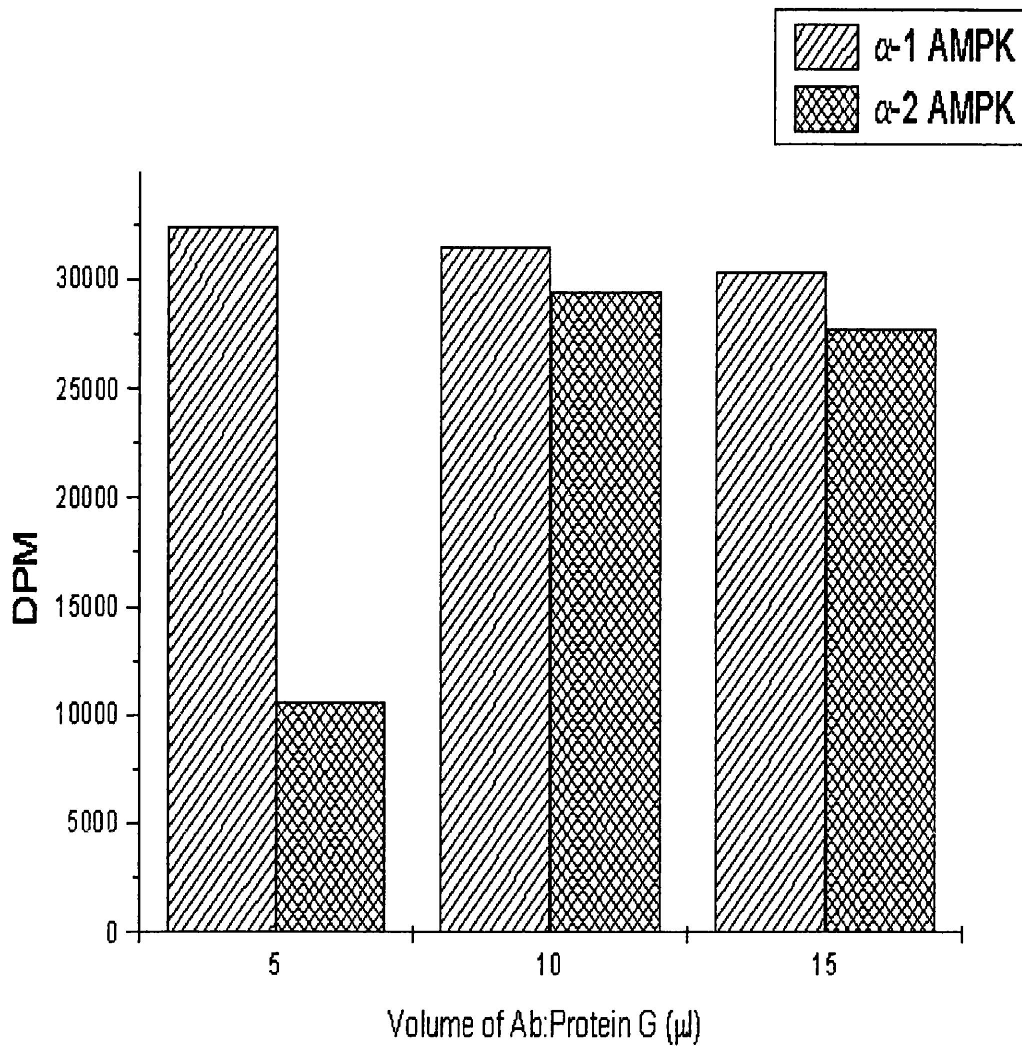
Antisera raised in sheep against both the α-1 and α-2 subunits of AMPK was used to obtain a more highly purified sample of the kinase for use in measuring cardiac AMPK activity. Prior to use the antiserum was bound to protein G-sepharose to enable the recovery of immunoprecipitates by centrifugation. Pre-immune sheep serum also bound to protein G-sepharose was used to generate blank reaction values, as preliminary experiments showed that these gave slightly higher dpm values than samples immunoprecipitated with anti-AMPK antisera and assayed minus SAMS peptide.

Preliminary experiments were carried out in order to determine the reaction time course and the optimum volume of anti-AMPK antiserum required to immunoprecipitate all AMPK activity. The results of these experiments are shown in figures 3.4. and 3.5. Figure 3.4. shows that a small volume (5μl) of anti- α-1 serum was adequate to immunoprecipitate the maximum kinase activity. The activity of α-2 AMPK increased with the inclusion of extra antiserum. To ensure the recovery of all AMPK activity and for ease of washing the recovered pellets, 15μl of antiserum-protein G-sepharose was used in all further experiments. Figure 3.5. shows that the activity of both α-1 and α-2 AMPK complexes was linear with time for 30 minutes and subsequent assays were incubated for this period.

3.2. Effects of Alternative Substrates.

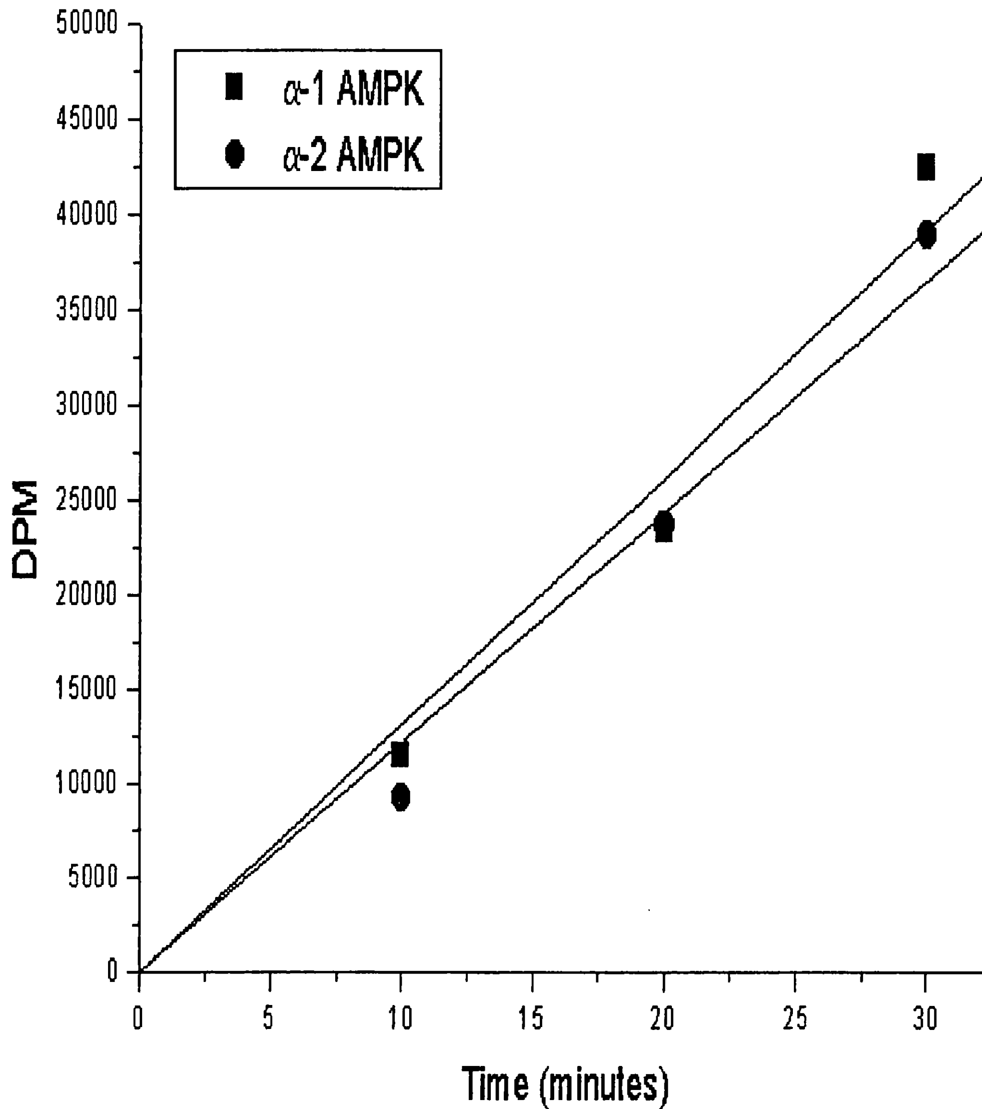
The availability of alternative substrates can alter the relative contribution of lipid and carbohydrate fuels to energy production. A glucose sparing effect is

Figure 3.4. Effects of Altering Antibody Concentration on α-1 and α-2 AMPK Activity.



In order to determine the optimum quantity of antibody required to extract all kinase activity, heart tissue was homogenised and AMPK activity immunoprecipitated with the indicated volume of Ab:Protein G. Assays were carried out in the presence of 200μM AMP. Values are disintegrations per minute for one heart sample.

Figure 3.5. Time Course of AMPK Assay.



AMPK was immunoprecipitated with 15μl of anti- α-1 or α-2 antiserum bound to protein G-sepharose and assayed with 200μM AMP. Reactions were stopped after 10, 20 or 30 minutes. The values shown are disintegrations per minute for a single heart sample.

observed when hearts are perfused with exogenous pyruvate and ketone bodies (Randle P.J. *et al.*, 1963). Increasing the supply of exogenous palmitate to isolated working rat hearts has been shown to inhibit both glycolysis and glucose oxidation and enhance rates of fatty acid oxidation (Saddik M and Lopaschuk GD, 1991).

This suppression of glucose utilisation in response to increased provision of fatty acids can be explained by the 'glucose fatty acid cycle' model in which glucose uptake as well as the activities of PFK1, PFK2 and PDH are inhibited. In order to inhibit carbohydrate metabolism fatty acids must first enter the mitochondria and be oxidised. For this to occur fatty acid fuels must initially signal their availability in order to overcome the glucose stimulated increase in malonyl-CoA concentration and relieve inhibition of CPT1.

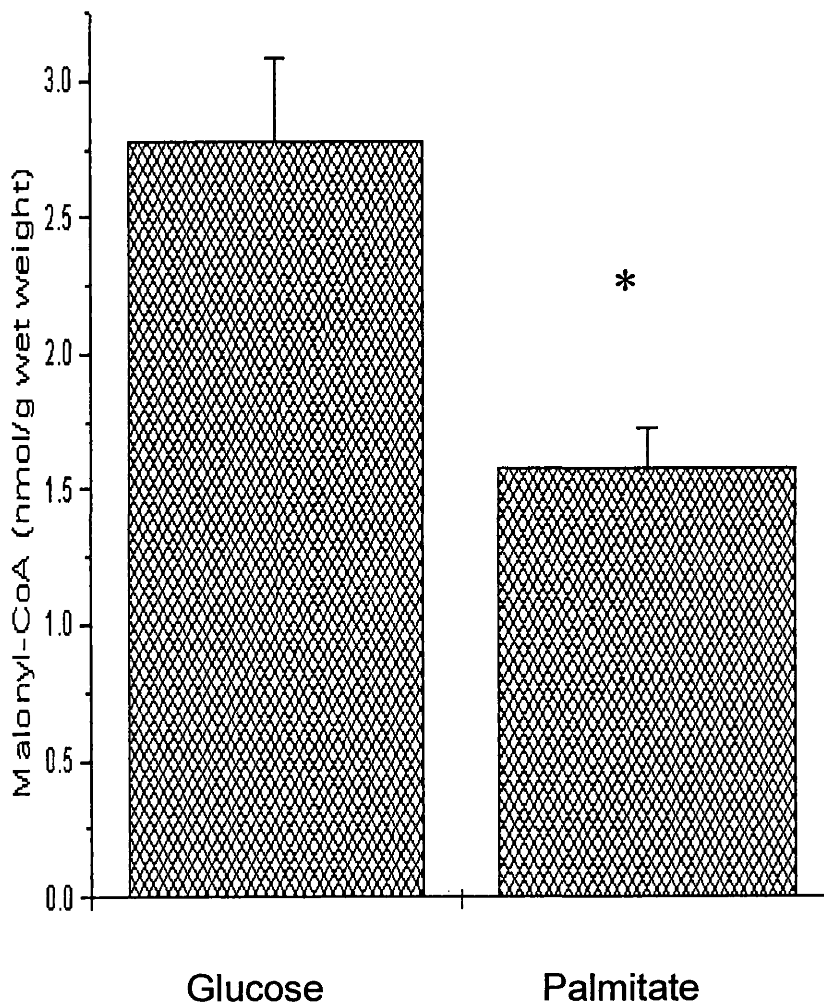
The following section outlines results from experiments carried out to test the hypothesis that fatty acids are able to signal their availability by activating the AMPK cascade in response to AMP generated by the fatty acyl-CoA synthase reaction. This stimulation of AMPK would result in the phosphorylation and inhibition of ACC, decrease malonyl-CoA content and increase rates of β-oxidation.

3.2.1. Effect of Palmitate on Cardiac Malonyl-CoA.

Rates of fatty acid oxidation in the heart can be correlated with tissue malonyl-CoA concentration under a range of conditions due to the ability of this effector to inhibit CPT1, and therefore decrease rates of fatty acid translocation into mitochondria. Supplying palmitate to perfused hearts (Awan MM and Saggerson ED, 1993) and isolated cardiac myocytes (Hamilton C and Saggerson ED, 2000) results in a lowering of the tissue malonyl-CoA concentration and increased rates of fatty acid oxidation.

In order to test the theory that palmitate is able to inhibit ACC activity via an activation of AMPK it was necessary to show that decreases in tissue malonyl- CoA could be observed in the palmitate perfused rat hearts utilised in this study. As can be seen in figure 3.6., in agreement with previous studies the inclusion of 0.5mM palmitate into heart perfusion media resulted in a significant ($p<0.01$) decrease in cardiac malonyl-CoA concentration. The

Figure 3.6. Effects of Palmitate on Malonyl-CoA Levels.



Hearts were perfused with 5mM glucose and 2% BSA ± 0.5mM palmitate. Malonyl-CoA concentrations are shown as the mean ± SEM of 5 independent preparations.

* indicates p< 0.01 for the effect of palmitate.

concentrations of malonyl-CoA observed in this study are in agreement with those measured previously by Awan and Saggerson in 1993.

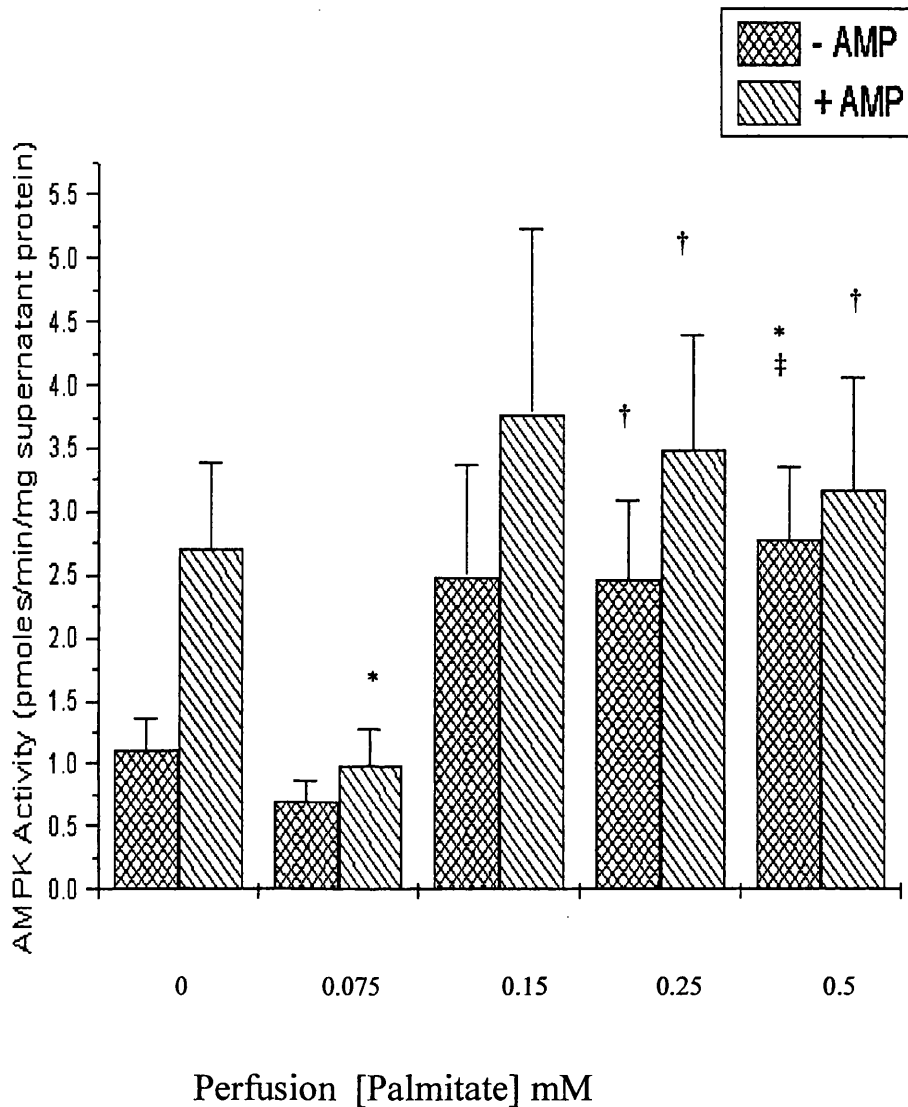
3.2.2. AMPK Activity.

Both α-1 and α-2 AMPK subunits are expressed by the rat heart, with α-2 complexes responsible for 70-80% of kinase activity (Cheung PCF *et al.*, 2000). Measurements of AMPK activity were made for complexes containing both subunits after a one hour perfusion followed by immunoprecipitation under conditions designed to maintain the phosphorylation state of the enzyme. Assays were performed in the presence and absence of 200μM AMP for 30 minutes on extracts from hearts perfused with 0, 0.075, 0.15, 0.25 and 0.5mM palmitate bound to 2% albumin. AMPK activity is expressed as pmol/min/mg of 13,000g supernatant protein, ie relative to the protein concentration directly before immunoprecipitation.

3.2.2.1. Effects of Palmitate on α-1 AMPK.

Figure 3.7. shows the effect of increasing palmitate concentration on AMPK α-1 activity. Palmitate at 0.5mM significantly ($p<0.05$) increased α-1 AMPK activity when measured in the absence but not in the presence of 200μM AMP. An unexpected result of these experiments was the inability of AMP to stimulate α-1 AMPK in immunoprecipitates from palmitate perfused hearts. A significant stimulation of activity by AMP ($p<0.02$, paired test) was observed only in hearts perfused with glucose as the sole substrate. The possibility that the lack of stimulation was due to the quality of AMP used in the assay can be eliminated as α-2 AMPK assays, where AMP stimulation always occurred, were always run in parallel. It has previously been reported that rat liver α-1 AMPK complexes are less sensitive to the allosteric effect of AMP than complexes containing the α-2 subunit (Salt I.P. *et al.*, 1998). Recent evidence has also shown that phosphorylation of the β-1 subunit of AMPK can alter the sensitivity of the enzyme to AMP activation (Warden SM *et al.*, 2001). Whether palmitate promotes phosphorylation of such a site remains to be determined. The AMP-dependence of the kinase can also be altered by the γ- subunit present in the heterotrimer, with complexes containing γ-3 being

Figure 3.7. Effects of Palmitate on AMPK α-1 Activity.



Hearts were perfused with 5mM glucose, 2% BSA and the indicated concentration of palmitate. AMPK α-1 activity was immunoprecipitated and measured in the presence or absence of 200μM AMP. Activity is shown as the mean ± SEM of between 5 and 7 independent perfusions.

* indicates $p < 0.05$ for the effect of palmitate versus zero palmitate under same assay condition. AMP stimulation was not significant, except for hearts perfused in the absence of fatty acid ($p<0.02$, paired test). † and ‡ indicate p< 0.05 and 0.01 respectively versus the effect of 0.075mM palmitate.

the least and those containing γ-2 the most AMP dependent. However kinase activity measurements have shown the major γ- subunit in heart tissue is the γ-1 (80-90%) with the rest due to γ-2 (Cheung PCF *et al.*, 2000). In heart the α-1γ-1 complexes would be expected to be the least AMP dependent. However no evidence exists to indicate any selective association between subunit isoforms.

A second unexpected finding of this set of experiments was the significant ($p<0.05$) decrease in α-1 AMPK activity in hearts perfused with the lowest (0.075mM) concentration of palmitate relative to controls. This decrease was significant only when assays were performed in the presence of 200μM AMP. One possible explanation for this reduction in kinase activity is that it was due simply to the inability of AMP to activate the kinase after perfusion with palmitate.

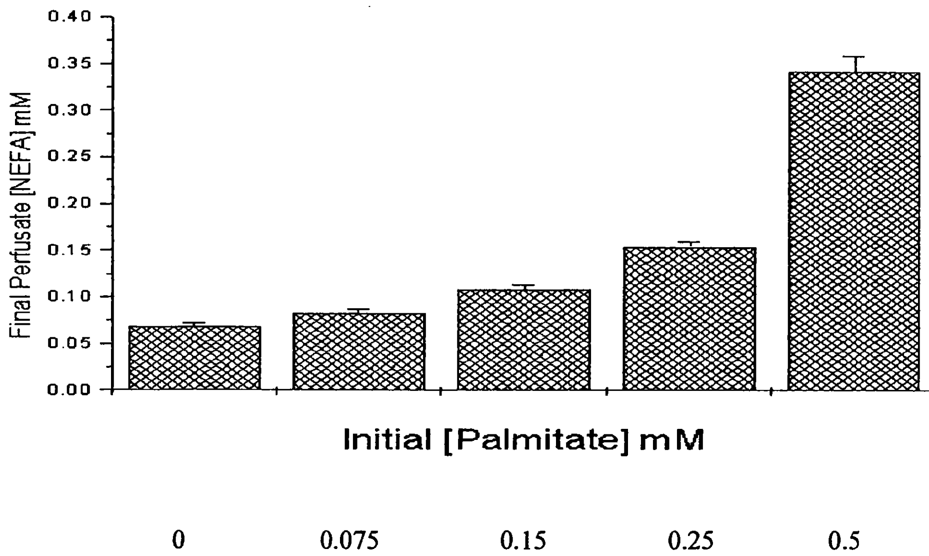
Figure 3.8.A. shows the final concentration of fatty acid against initial palmitate concentration in the perfusion media of hearts after an hour of perfusion with a range of [palmitate]. Figure 3.8.B. illustrates the loss/gain of NEFA at each concentration. Hearts perfused with palmitate concentrations above 0.15mM showed net uptake of fatty acids from the perfusion media, whilst hearts perfused with glucose as the sole substrate released NEFA. At a concentration of 0.075mM palmitate there was little net loss or gain of fatty acid suggesting the hearts were close to 'NEFA balance'. Whether this observation is related to the drop in kinase activity measured in hearts perfused at this concentration of palmitate is not clear and if so would indicate a sensitivity of α-1 AMPK to the overall metabolism of fatty acid (lipolysis as well as oxidation).

3.2.2.2. Effects of Palmitate on α-2 AMPK.

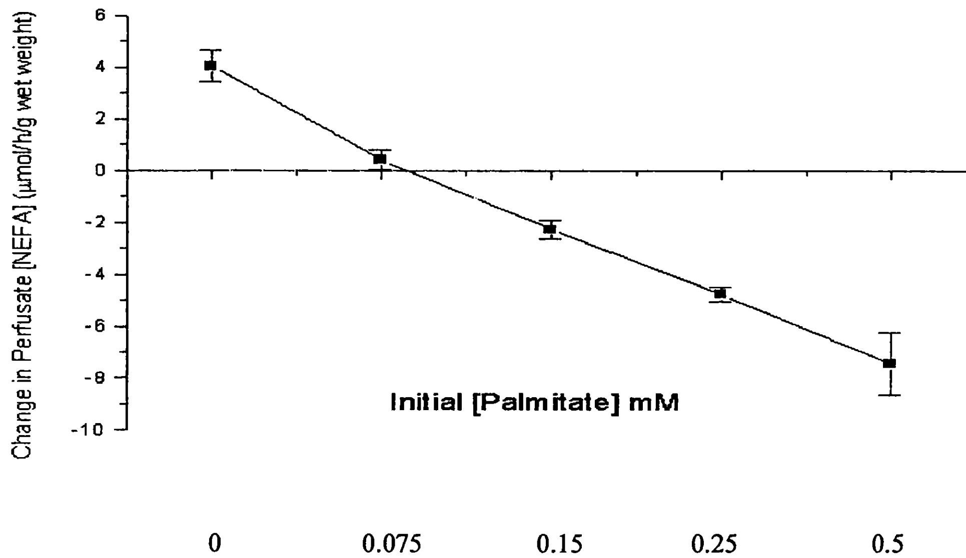
Figure 3.9. shows the effect of perfusion with 0, 0.075, 0.15, 0.25 and 0.5mM palmitate on the activity of α-2 AMPK activity. Unlike α-1, the effect of 200μM AMP to stimulate kinase activity was significant ($p< 0.05$ at 0, 0.075 and 0.25mM palmitate, $p< 0.01$ at 0.15 and 0.5mM palmitate, paired tests) under each perfusion condition. Activation of α-2 AMPK by palmitate was more graded than the activation of α-1 complexes, with the effect being significant at perfusion concentrations of 0.25 and 0.5mM. Although not significant, α-2

Figure 3.8. Perfusate NEFA After 1hour Perfusion.

A.



B.

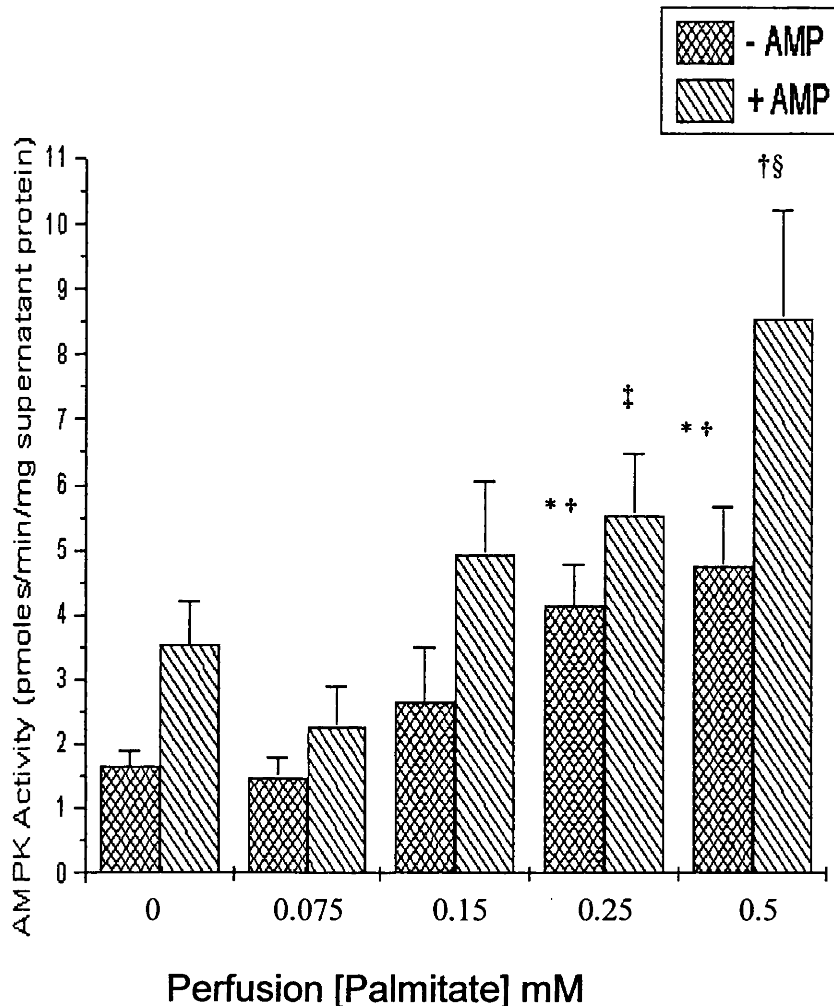


A. Shows the perfusate fatty acid concentration at the end of a one hour recirculating perfusion.

B. Shows the net utilisation or release of fatty acids for each initial palmitate concentration.

Values are the mean ± SEM for 4-7 independent perfusions.

Figure 3.9. Effects of Palmitate on AMPK α-2 Activity.



Hearts were perfused with 5mM glucose, 2% BSA and the indicated concentration of palmitate. AMPK α-2 activity was immunoprecipitated and measured ± 200μM AMP. Activity is shown as the mean ± SEM of 6 or 7 independent perfusions.

§ and * indicate p< 0.02 and 0.01 respectively for the effect of palmitate versus zero palmitate, ‡ and † are p< 0.02 and 0.01 versus 0.075mM palmitate, under the same assay conditions. The effect of AMP was significant in each data set (p< 0.05 for 0, 0.075 and 0.25mM palmitate, p< 0.01 for 0.15 and 0.5mM palmitate, paired tests).

like α-1 kinase activity tended to be reduced at a perfusion palmitate concentration of 0.075mM.

Previous studies have shown that complexes containing α-2 AMPK account for 70-80% of the kinase activity in freshly isolated rat heart tissue (Cheung PCF *et al.*, 2000). Assays carried out on the perfused rat hearts show that α-2 activity accounted for between 57 and 74% of the total activity depending on the perfusion condition, the contribution of α-2 being higher in hearts exposed to 0.5mM palmitate. The hearts utilised in the Cheung experiments were freshly isolated and could therefore have been exposed to high plasma [NEFA] explaining the high α-2/α-1 ratio. The discrepancies could also due to the specificity of each isoform for the different peptide substrate used (AMARA versus SAMS). AMPK complexes containing α-1 subunits phosphorylate SAMS peptide at approximately 75% of the rate for AMARA phosphorylation, whilst α-2 complexes phosphorylate SAMS at 50% of the rate of the AMARA substrate (Woods A *et al.*, 1996). The percentage contribution to total kinase activity of each α isoform would therefore be expected to vary depending on the peptide substrate utilised.

Previous studies using working rat hearts have failed to observe changes in AMPK activity on increasing the palmitate concentration from 0.4 to 1.2mM (with 3% BSA) (Sakamoto J *et al.*, 2000; Longnus SL *et al.*, 2001). The lack of AMPK activation observed in these studies could be due to the different methods employed for isolation of AMPK (PEG precipitation versus immunoprecipitation). The composition of perfusion buffers was also slightly different, with lactate 0.5mM included in the Longnus study and glucose concentration raised from 5.5 to 20mM in the Sakamoto experiments. The results described here show that α-2 AMPK was activated by palmitate at 0.25mM (with 2% BSA). This represents a fatty acid/albumin molar ratio of 0.84. No further increase in kinase activity was observed on increasing palmitate to 0.5mM (BSA 2%) i.e. a molar ratio of 1.67. The lack of effect on AMPK in the two studies mentioned above could therefore be due to the kinase being activated at the lower 0.4mM palmitate, (fatty acid/albumin ratio =0.89) with no further stimulation occurring on increasing this ratio to 2.68.

3.2.2.3. Effect of PP2C Treatment on AMPK Activity.

The α subunit of AMPK heterotrimers contains the Thr-172 residue phosphorylated by the upstream AMPKK. Both α-1 and α-2 are readily dephosphorylated by PP2C in the presence of Mg^{2+} (Salt I.P. *et al.*, 1998). In order to determine whether the activation of AMPK in response to palmitate was due to increased protein phosphorylation, the effect of PP2C on kinase activity was tested.

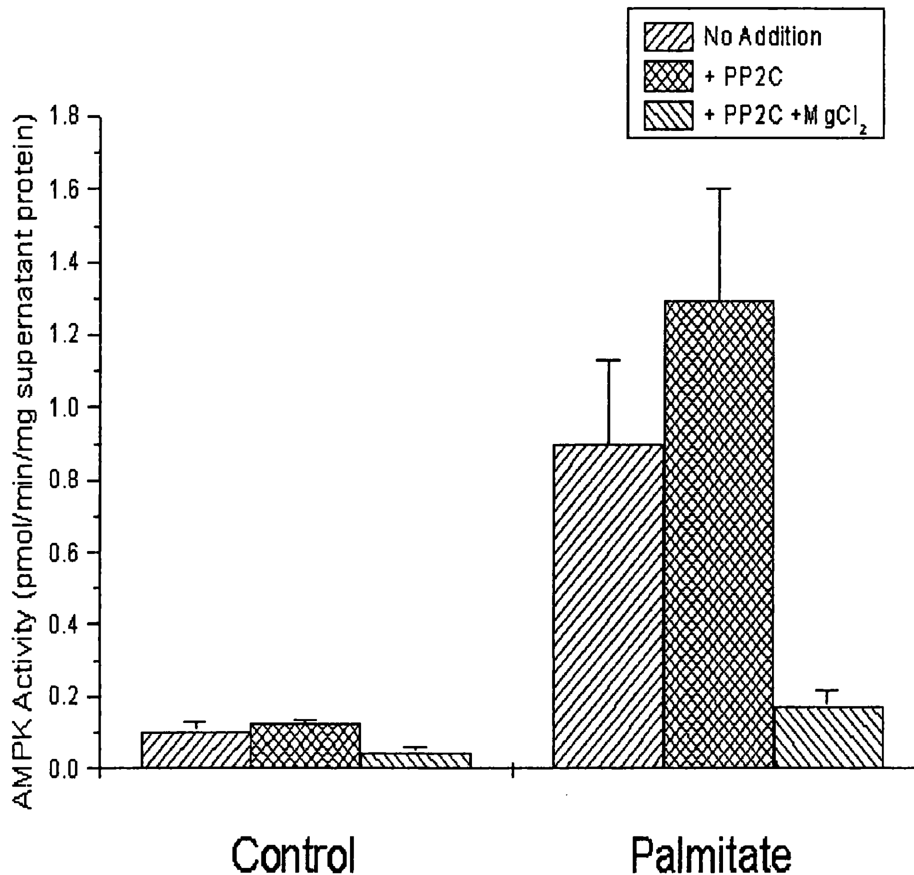
Figures 3.10. and 3.11. show the effect of PP2C treatment on α-1 and α-2 AMPK activities respectively. Kinase activity was decreased in tissue from both control and 0.5mM palmitate perfused hearts when incubated with 4μg PP2C in the presence of 10mM $MgCl_2$. Dephosphorylation of AMPK complexes containing both α subunits resulted in a reversal of the stimulation of activity due to palmitate, supporting the hypothesis that palmitate exerts its effect by increasing protein phosphorylation. The activity of both AMPK isoforms was lower in this set of experiments than those measured previously, and the degree of activation by palmitate higher. Loss of kinase activity could potentially be a result of the extra washing procedures involved in preparing immunoprecipitates for assay after phosphatase treatment, as well as the increased delay in assay commencement, (at least 90 minutes).

The reason for the higher degree of palmitate activation is unclear. It has been reported previously that dephosphorylated AMPK is less stable than the phosphorylated form on storage (Hawley SA *et al.*, 1996). The results here show that phosphorylation of both α-1 and α-2 AMPK was increased in tissue from hearts perfused with palmitate. Potentially the less phosphorylated AMPK immunoprecipitated from control hearts was unstable and activity was lost over time.

3.2.2.4. Effect of Palmitate on Thr-172 Phosphorylation.

Threonine-172 has been identified as the major site on both α catalytic subunits phosphorylated in response to activation of the upstream AMP-dependent AMPKK (Hawley SA *et al.*, 1996). To determine whether

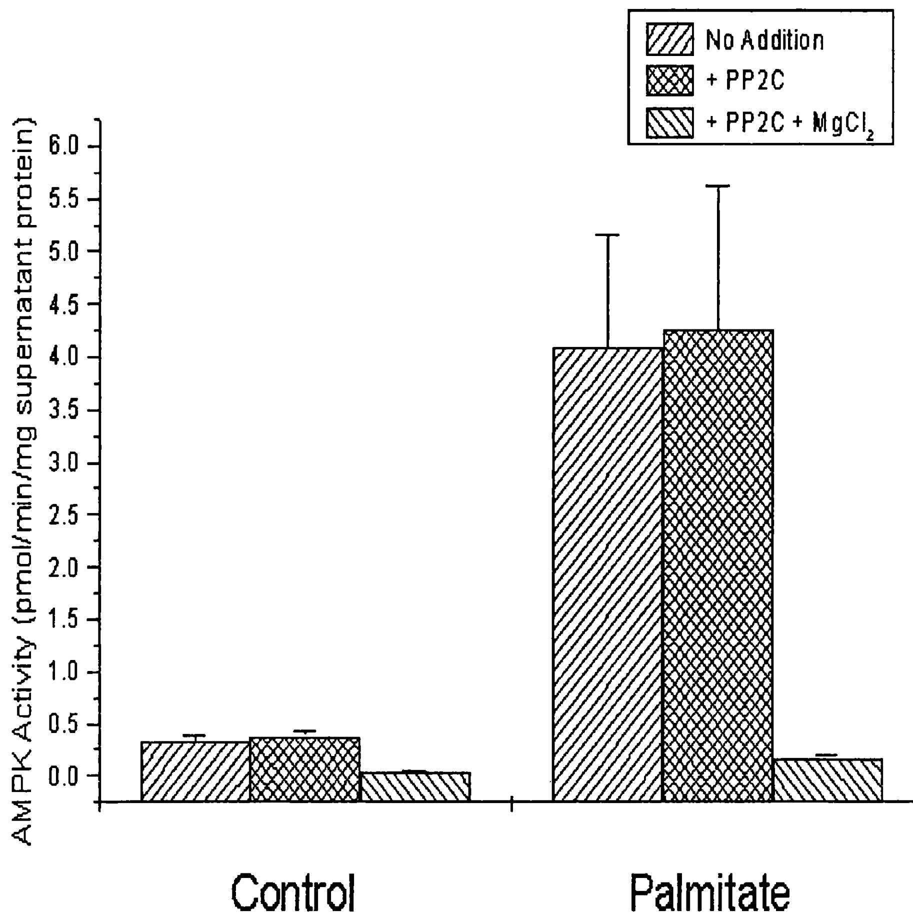
Figure 3.10. Effects of Protein Phosphatase 2C Treatment on α-1 AMPK Activity.



Hearts were perfused with 5mM glucose and 2% BSA ± 0.5mM palmitate. α-1 AMPK complexes were immunoprecipitated and assayed in the absence of AMP after a 30 minute incubation with no addition, or addition of PP2C ± 10mM $MgCl_2$.

Values are the mean ± SEM for 4 independent perfusions.

Figure 3.11. Effect of Protein Phosphatase 2C Treatment on α-2 AMPK Activity.



Hearts were perfused with 5mM glucose, 2% BSA ± 0.5mM palmitate. α-2 AMPK complexes were immunoprecipitated and assayed in the absence of AMP after a 30 minute incubation with no additions or with PP2C ± 10mM $MgCl_2$.

Values are the mean ± SEM of 4 independent perfusions.

phosphorylation of this residue was increased in response to palmitate, Dr. David Carling (MRC Clinical Sciences Group, Hammersmith carried out immunoblotting experiments. Samples of 13,000g supernatants (200μg of protein) were analysed by SDS-PAGE (7% acrylamide), transferred to PVDF and blotted with anti-phosphoAMPK antibody. The secondary antibody used was a donkey anti-rabbit conjugated to horseradish peroxidase and phosphorylated AMPK was visualised using a Boehringer ECL kit.

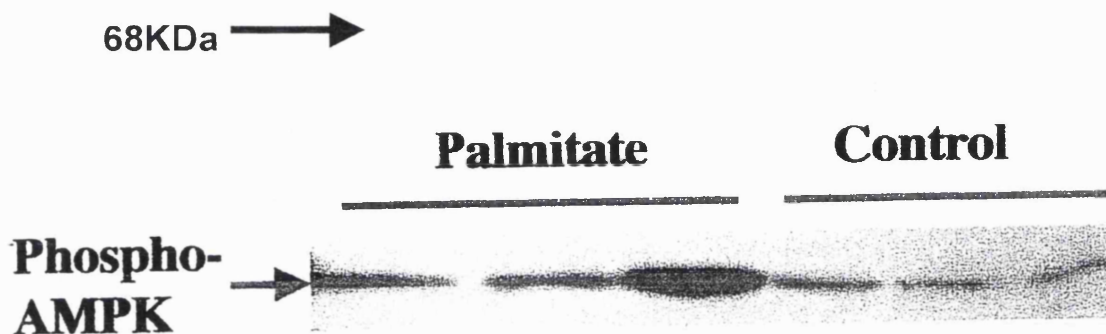
Figure 3.12. shows the results of immunoblotting on three control and three palmitate perfused hearts. Hearts perfused in the presence of 0.5mM palmitate tended to contain AMPK with increased phosphorylation at thr-172. Other sites have been identified on both α and β AMPK subunits that are phosphorylated by the upstream AMPKK (Mitchelhill K.I. *et al.*, 1997; Stein SC *et al.*, 2000). The possibility that additional, unidentified protein kinases may also regulate the activity of AMPK has also been suggested. It remains possible that palmitate covalently modifies the kinase at additional sites and in response to alternative AMPK kinases.

3.2.3. Effect of Palmitate on Adenine Nucleotide Content.

In order to determine whether the palmitate-induced activation of AMPK was due to an increase in the tissue content of AMP, total adenine nucleotide content was measured. Tissue from hearts perfused without and with 0.075mM and 0.5mM palmitate were subject to acid extraction as described in section 2.5.1. and adenine nucleotides measured by HPLC analysis. Figure 3.13. shows the tissue content of ATP, ADP and AMP in the three heart groups. No significant difference in total AMP concentration or in the AMP/ATP ratio was observed after perfusion with either palmitate concentration compared to control hearts (AMP:ATP was, 0.071± 0.011 in control, 0.047± 0.006 and 0.054 ± 0.005 for hearts perfused with 0.075 and 0.5mM palmitate respectively). Ratios of ATP/AMP were almost identical to those measured previously in aerobically perfused hearts using a similar perfusion system (Beauloye C *et al.*, 2001).

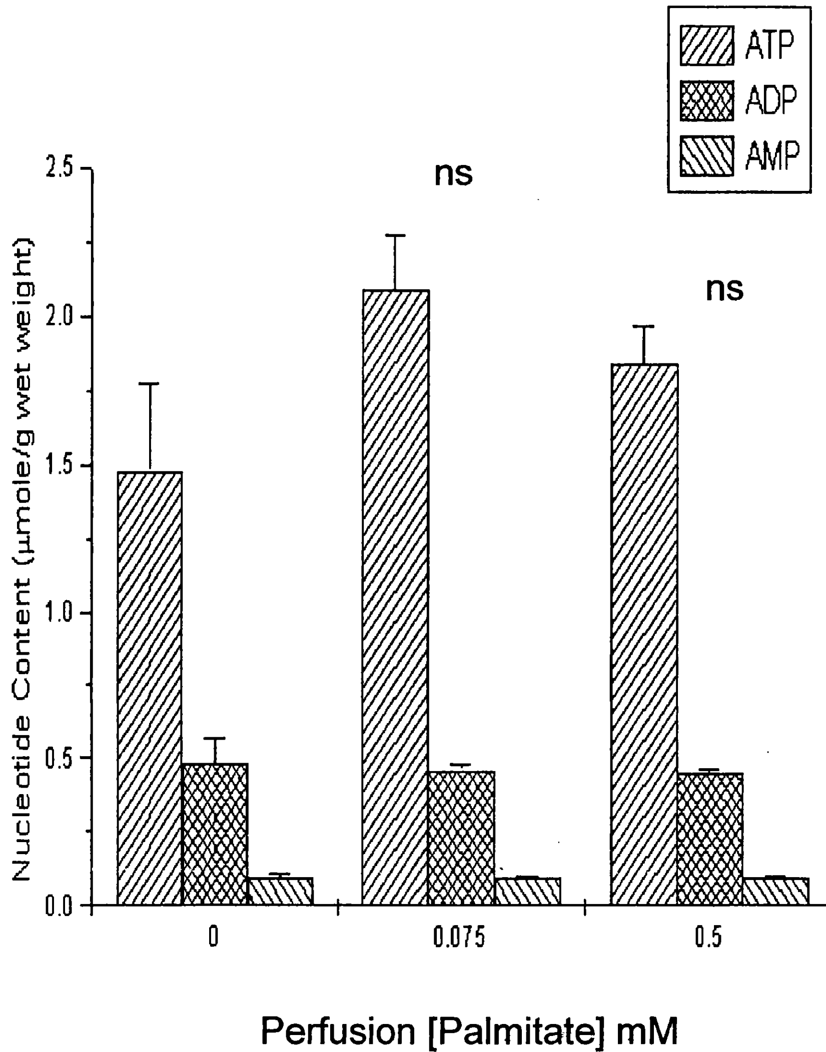
Prior to the commencement of this study increased phosphorylation/ activation of AMPK had been believed to occur only under conditions which increased the cellular AMP: ATP. One report that observed increased AMPK

Figure 3.12. Effect of Palmitate on AMPK Thr172 Phosphorylation.



Hearts were perfused with 5mM glucose, 2% BSA ± 0.5mM palmitate followed by extraction and immunoblotting using an anti-phosphoAMPK antibody which recognises the site surrounding phospho-Thr172 on both α-1 and α-2 catalytic subunits . The results show 3 separate hearts for each perfusion condition. Band intensities are as follows from left to right: 78006,83265, 196932, 53083, 32210 and 24764, giving a 3.26 increase in intensity due to palmitate. This increase is not however significant ($p<0.2$) and further blots need to be performed.

Figure 3.13. Effects of Palmitate on Cardiac Adenine Nucleotide Content.



Hearts were perfused for one hour with 5mM glucose, 2% BSA and the indicated concentration of palmitate. Adenine nucleotide content was measured by HPLC analysis following TCA extraction.

Values are the mean ± SEM of 6 or 7 independent perfusions. ns = not significantly different from controls for all adenine nucleotides.

activity in exercising red quadriceps muscle without an alteration of the AMP/ATP ratio explained this anomaly by the delay (90-120s) between exercise termination and tissue freezing during which the nucleotide levels returned to control levels (Winder WW and Hardie DG, 1996). Later studies on AMPK indicate that changes in the phosphorylation state of the kinase can occur independently of alterations in tissue AMP/ATP ratios. Leptin has been shown to activate α-2 AMPK in skeletal muscle (Minokoshi Y. *et al.*, 2002), treatment of perfused hearts with insulin decreases AMPK activity in aerobic hearts and prevents activation of the kinase during ischaemia (Beauloye C *et al.*, 2001) without detectable changes in the AMP/ ATP ratio.

These results do not support the theory that AMP produced by the fatty acyl-CoA synthetase reaction is responsible for the palmitate-induced activation of AMPK activity. As the total oxidation of one molecule of palmitate results in a net yield of 129 ATP this result is perhaps not surprising. Most of the palmitoyl-CoA synthetase in heart is found on the outer mitochondrial membrane (DeJong JW and Hülsman WC, 1970) and it is possible that localised increases in AMP occur, which cannot be detected in whole tissue extracts. Further experimental work, perhaps using specific inhibitors of fatty acid activation could be carried out to determine the contribution of this reaction in stimulating AMPK.

Stimulation of AMPKK activity in response to palmitoyl-CoA has also been reported (Carling D *et al.*, 1987). It seems unlikely that the stimulation of AMPK observed in response to palmitate was due to long chain acyl-CoA. Although not measured in this study, no accumulation of long chain CoA esters was observed in isolated cardiac myocytes in which malonyl-CoA concentration was reduced after incubation with palmitate (Hamilton C and Saggerson ED, 2000). The mechanism by which palmitate regulates the activity of protein kinases/ phosphatases acting on AMPK remains to be resolved.

3.2.4. Effects of Palmitate on ACC Phosphorylation and Activity.

ACC2 is the major ACC isoform expressed in cardiac tissue (Bianchi A *et al.*, 1990) with the 265-kDa isoform expressed at a lower level (Kudo N *et al.*,

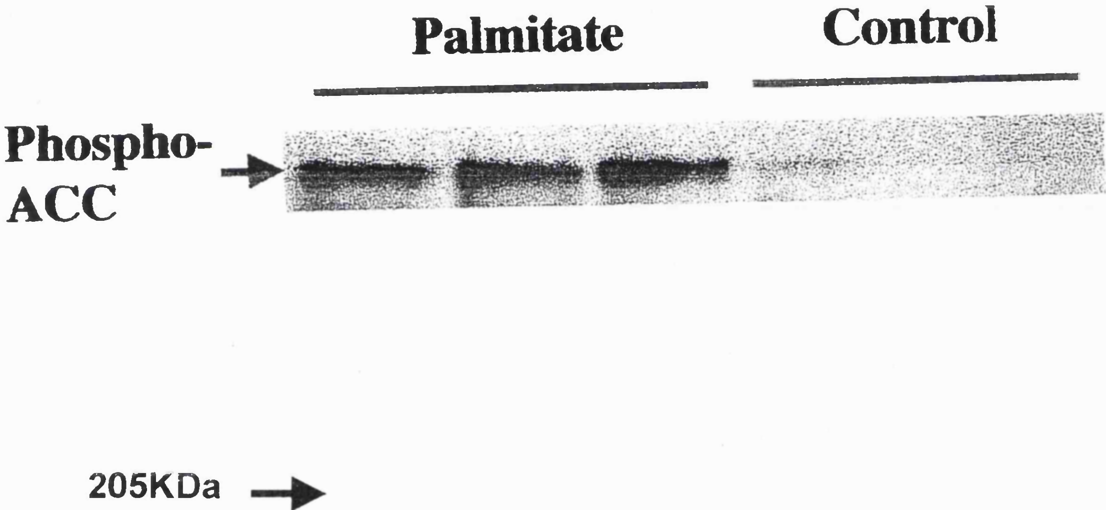
1995). ACC2 purified from heart muscle is phosphorylated and inactivated after incubation with AMPK (Dyck JRB *et al.*, 1999). In order to determine the effect of the increase in AMPK activity due to palmitate, experiments were carried out to measure the activity and phosphorylation state of ACC.

Immunoblotting experiments were carried out at the MRC Clinical Sciences Centre at Hammersmith Hosp. by Dr. David Carling. Heart tissue was homogenised and samples containing 200μg of 13,000g supernatant protein were analysed by SDS-PAGE (7% acrylamide), transferred to PVDF and blotted with an anti-phosphoACC antibody which recognises the sequence corresponding to amino acids 73-85 of rat ACC1 [HMRSSMS(PO_4)GLHLVK]. The secondary antibody used was donkey anti-rabbit conjugated to horseradish peroxidase and phospho-ACC protein was detected using a Boehringer ECL kit. Figure 3.14. shows the phosphorylation state of samples from three palmitate and three control hearts. Hearts exposed to 0.5mM palmitate during perfusion showed greatly increased phosphorylation of ACC, consistent with an activation of AMPK.

ACC activity was measured both in 100,000g supernatants and resuspended pellets. Despite reports that ACC2 is anchored to mitochondria (Abu-Elheiga L *et al.*, 2000) no activity was detected in particulate fractions. For this reason comparisons of ACC activity between control and palmitate perfused hearts were made using 100,000g supernatants. Assays were performed in the presence and absence of 10mM citrate. As ACC2 is highly dependent on citrate, the citrate-dependent (total activity minus activity in the absence of citrate) was taken to be due to authentic ACC. Carboxylase activity measured in the absence of citrate is not relevant to the regulation of malonyl-CoA levels, and is likely due to the action of propionyl-CoA carboxylase and/ or pyruvate carboxylase which are present in heart mitochondria (Thampy KG, 1989).

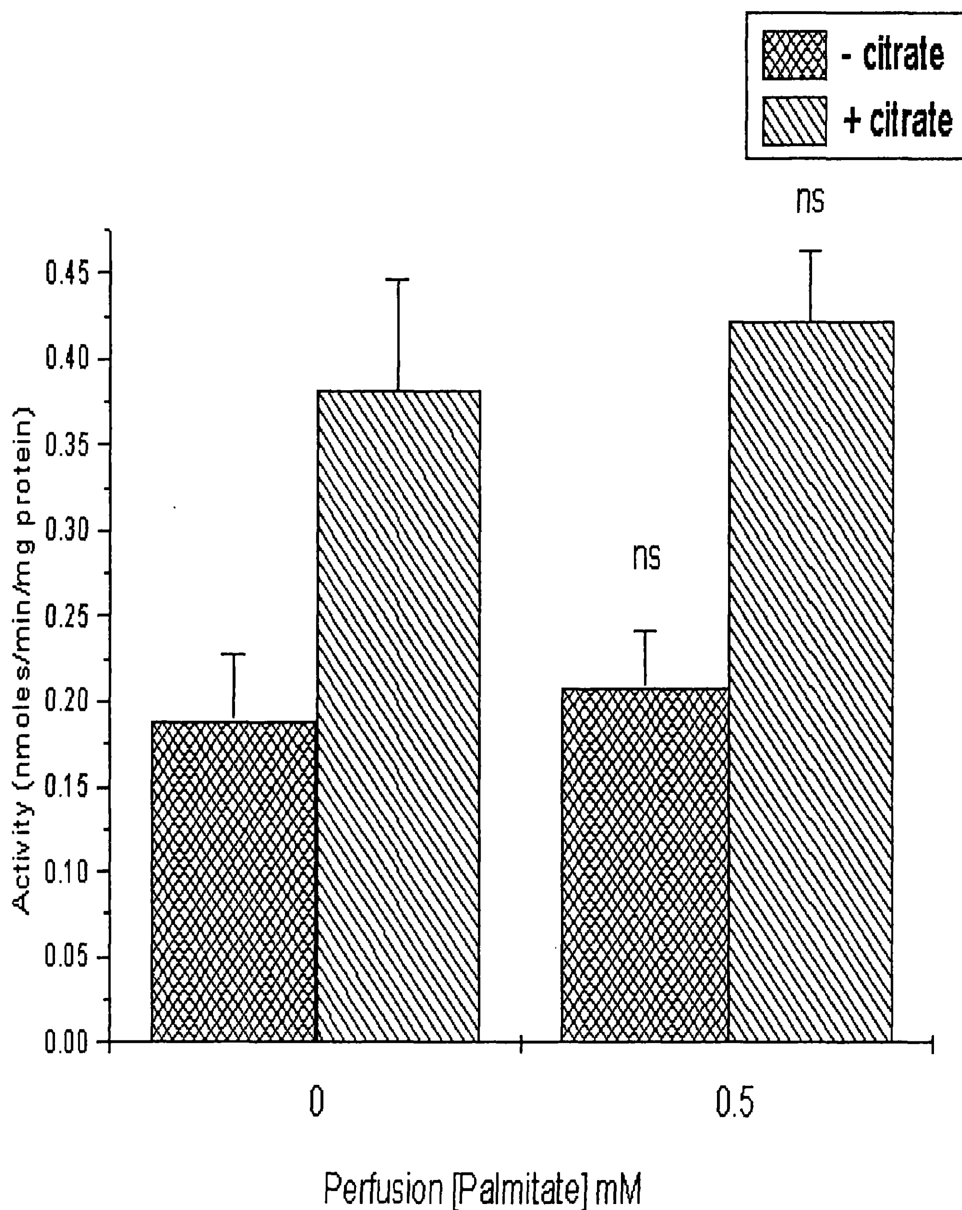
Figure 3.15. shows the result of perfusion with palmitate on ACC activity. Despite an expectation that ACC activity would decrease in hearts perfused with 0.5mM palmitate, no significant difference in total or citrate-dependent activity was measured compared to control hearts. Citrate-independent activity accounted for approximately 50% of the total in these assays, presumably as a result of mitochondrial breakage due to the hearts being

Figure 3.14. Effect of Palmitate on ACC Phosphorylation.



Hearts were perfused with 5mM glucose, 2% BSA ± 0.5mM palmitate, followed by extraction and immunoblotting using anti-phospho ACC antibody. Results shown are for 3 separate hearts at each perfusion condition.

Figure 3.15. Effects of Palmitate on ACC Activity.



Hearts were perfused with 5mM glucose, 2% BSA and the indicated concentration of palmitate. Activities are the mean ± SEM for 6 independent preparations. ns = not significantly different from controls.

freeze-clamped prior to the extraction process. ACC activity in this study was comparable to that previously measured in 100,000g supernatants of heart and cardiac myocytes (Awan MM and Saggerson ED, 1993) and in working rat hearts using dialysed 40,000g supernatants (Goodwin GW and Taegtmeyer H, 1999). Other researchers have assayed ACC activity in 6% PEG pellets. Measurements from aerobic hearts made using this extraction method give activities that are over 10 times higher than those measured here, and vary from between 6.16 nmol/min/mg protein (Sakamoto J *et al.*, 2000) to approximately 20 nmol/min/mg protein (Kudo N *et al.*, 1995) when assayed in the presence of 10mM citrate.

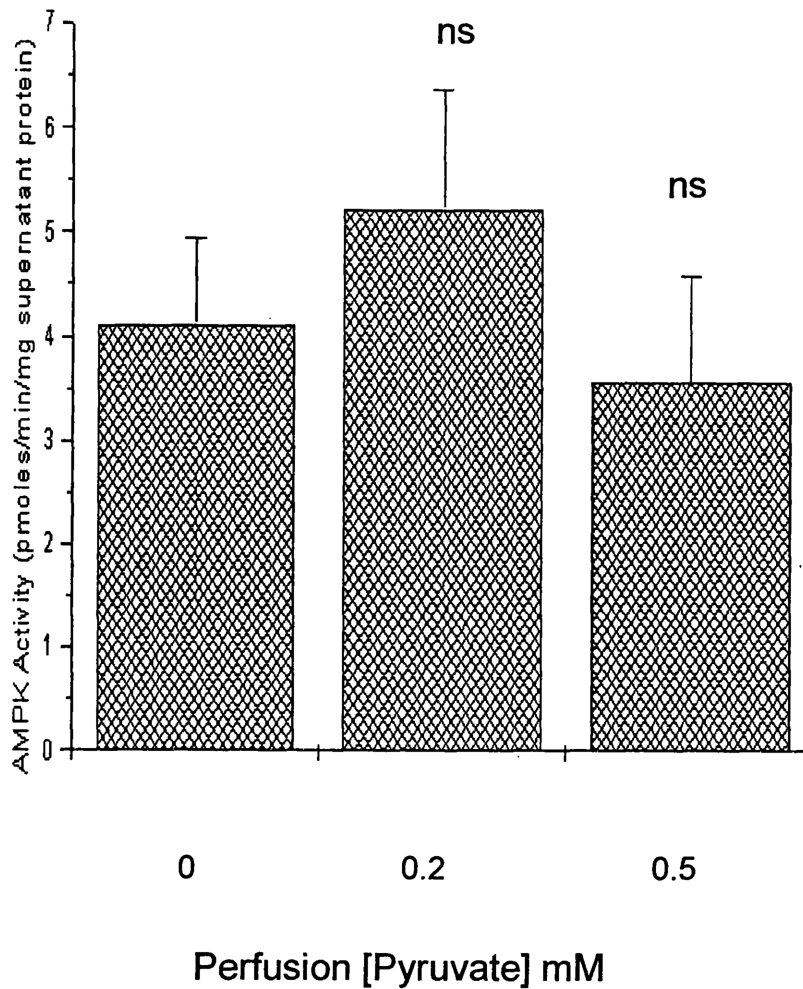
In the developing rabbit heart correlations between ACC activity and malonyl-CoA/ fatty acid oxidation have previously been seen (Lopaschuk GD *et al.*, 1994; Dyck JRB *et al.*, 1998), with inhibition of ACC shown to correspond to increases in AMPK expression and activity (Makinde AO *et al.*, 1997). Fatty acid oxidation increases in perfused hearts during ischaemia, an event associated with decreased levels of malonyl-CoA and increased AMPK activity (Kudo N *et al.*, 1995). These results were not correlated with changes in the activity of ACC. Although the researchers reported a decrease in total (measured with 10mM citrate) ACC activity, this occurred at the end of a period of reperfusion i.e. subsequent to decreases in malonyl-CoA. Also, although total carboxylase activity decreased in the study by Hudo *et al*, the citrate-dependent proportion of total ACC activity was actually slightly increased.

3.2.5. Effects of Pyruvate on AMPK Activity.

The observation that AMPK is activated in response to perfusion with palmitate appears to be contrary to the proposed role of the kinase as responding to situations of metabolic stress and fuel deprivation. In order to determine whether this activation was specific to fatty acid fuels or a more general response to excess fuel, perfusions were carried out which included 0.2mM or 0.5mM pyruvate with 5mM glucose.

Figure 3.16. shows the effect of pyruvate perfusion on α-2 AMPK activity measured in the presence of 200μM AMP. As can be seen no significant difference was observed in kinase activity after perfusion with 0.2 or 0.5mM

Figure 3.16. Effects of Pyruvate on α-2 AMPK Activity.



Hearts were perfused with 5mM glucose, 2% BSA and the indicated concentration of sodium pyruvate. α-2 complexes were immunoprecipitated and assayed with 200μM AMP.

Values are the mean ± SEM of 6 independent perfusions. ns indicates no significant differences versus zero pyruvate.

pyruvate compared to control hearts. Therefore it is likely that AMPK is not activated in response to high substrate availability which would have extended its description as a 'fuel gauge' as a regulator at both ends of the energy spectrum. In keeping with its role in regulating enzymes of lipid metabolism, it appears from these results that the kinase is sensitive only to lipid fuels.

3.3. Effects of Hormones.

3.3.1. Insulin.

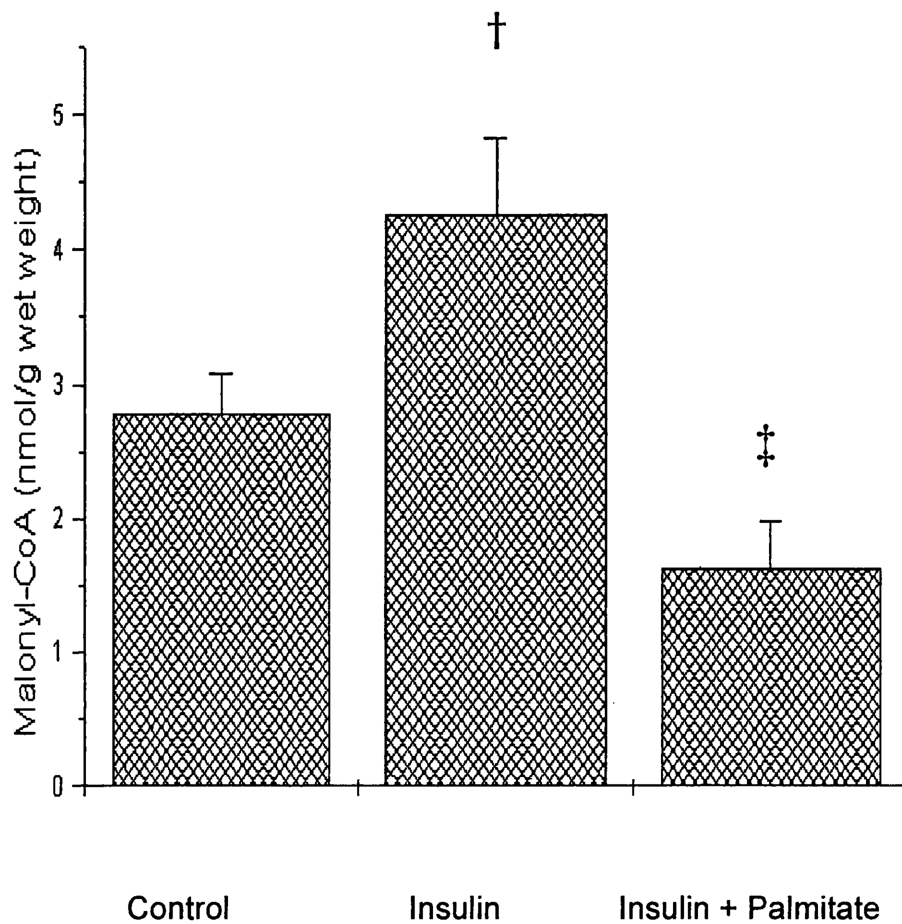
Insulin exposure signals the fed state and results in the appropriate alteration in fuel utilisation, an effect that is tissue specific. Addition of insulin (100μU/ml) to the perfusion media of working rat hearts increases the contribution of glucose derived acetyl-CoA to the citric acid cycle, with a simultaneous reduction (71% to 47%) in the contribution of palmitate when palmitate is supplied at 0.4mM. This insulin effect was overcome on increasing the palmitate concentration to 1.2mM (Sakamoto J *et al.*, 2000). Due to its ability to inhibit fatty acid oxidation and its reported inhibition of AMPK activity in hepatoma cells (Witters LA and Kemp BE, 1992) it was therefore of interest to this study to determine the effect of insulin on the activities of both ACC and AMPK in the perfused heart.

3.3.1.1. Effect of Insulin on Cardiac Malonyl-CoA.

The decreased rate of palmitate oxidation in insulin-treated hearts has been proposed previously to be due to inhibition of CPT1 by malonyl-CoA, the concentration of which has been shown to increase after insulin exposure (Awan MM and Saggerson ED, 1993). The same study also showed that the effect of insulin on cardiac malonyl-CoA, like rates of fatty acid oxidation could be overcome by the addition of palmitate (0.5mM) to the perfusion media.

In order to determine that the perfused hearts utilised for the assay of enzyme activities were responsive to insulin, measurements of tissue malonyl-CoA concentration were made. As can be seen from figure 3.17. in agreement with previous reports the inclusion of 10nM insulin in the heart perfusion

Figure 3.17. Effects of Insulin on Malonyl-CoA Concentration.



Control Insulin Insulin + Palmitate

Hearts were perfused with 5mM glucose, 2% BSA and where indicated palmitate (0.5mM) and/or insulin (10nM).

Values are the mean ± SEM of 5 or 6 independent perfusions. † indicates $p < 0.05$ for the effect of insulin. ‡ indicates $p < 0.01$ for the effect of palmitate.

media resulted in a significant ($p<0.05$) increase in malonyl-CoA concentration, an effect that was reversed by 0.5mM palmitate. These tissue malonyl-CoA concentrations are comparable to those previously measured in the study of Awan and Saggerson under similar perfusion conditions.

3.3.1.2. Effects of Insulin on AMPK Activity.

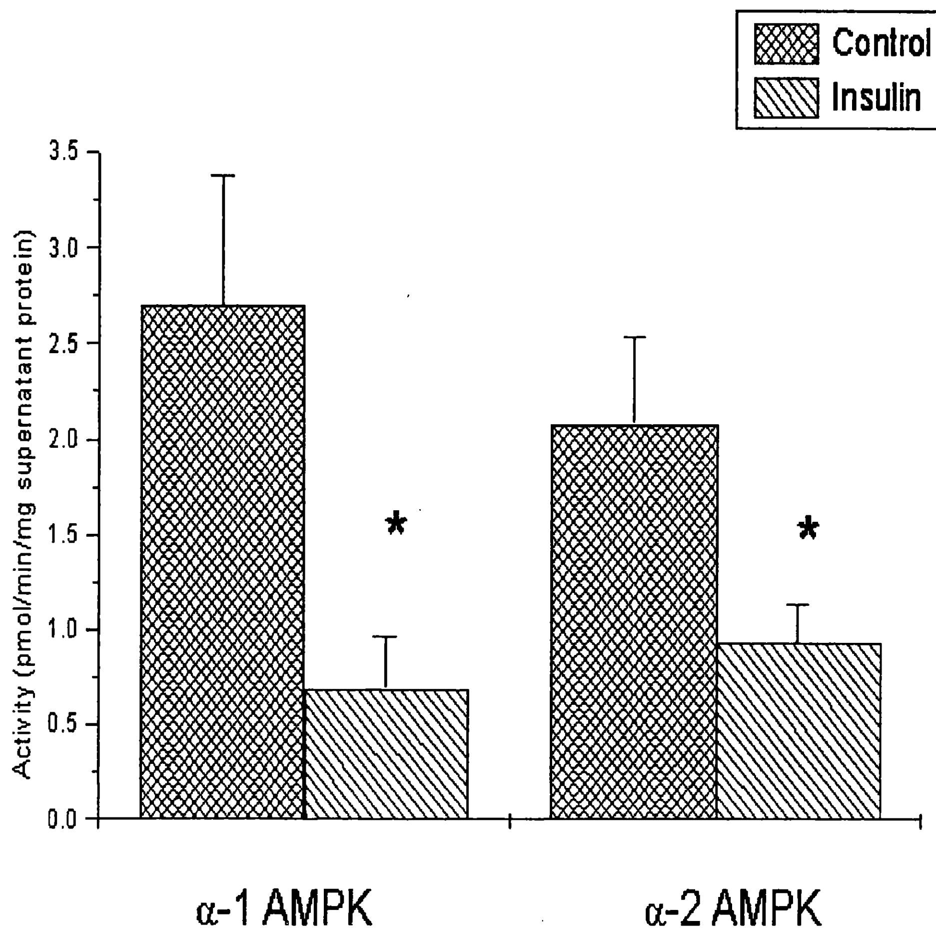
To test the theory that insulin increases malonyl-CoA by stimulating ACC activity via an inactivation of AMPK, hearts were perfused with 10nM insulin in the presence or absence of 0.5mM palmitate. AMPK activity was measured after immunoprecipitation of complexes containing α-1 or α-2 catalytic subunits.

Figure 3.18. shows the effect on AMPK activity of the inclusion of 10nM insulin to the perfusion media of hearts with glucose as the sole substrate. Insulin treatment caused significant ($p<0.05$) decreases in both α-1 and α-2 AMPK activities of 81% and 55% respectively.

Figure 3.19. shows the effect of insulin on AMPK activity in hearts perfused with 5mM glucose plus 0.5mM palmitate. Under these perfusion conditions, no significant effect of insulin on AMPK activity was observed, i.e. the insulin induced decrease in AMPK activity was reversed by the inclusion of palmitate into the perfusion medium. This result is interesting with regard to the ability of palmitate to overcome the insulin stimulated increase in cardiac malonyl-CoA concentration.

In agreement with these results insulin has recently been shown to decrease AMPK activity in rat hearts perfused aerobically with 5mM glucose as the sole substrate, as well as antagonising its activation by ischaemia (Beauloye C *et al.*, 2001). In working rat hearts perfused with either 0.4mM or 1.2mM palmitate the addition of 100μU/ml insulin had no effect on AMPK activity relative to hearts perfused in the absence of the hormone (Sakamoto J *et al.*, 2000). The effect of insulin on AMPK activity in hearts perfused in the absence of fatty acid were not determined in the Sakamoto study. In contrast other researchers have shown decreases in AMPK activity in response to insulin in hearts perfused with 0.4mM palmitate. Increasing the insulin concentration from 100 to 1000μU/ml resulted in an inhibition of AMPK

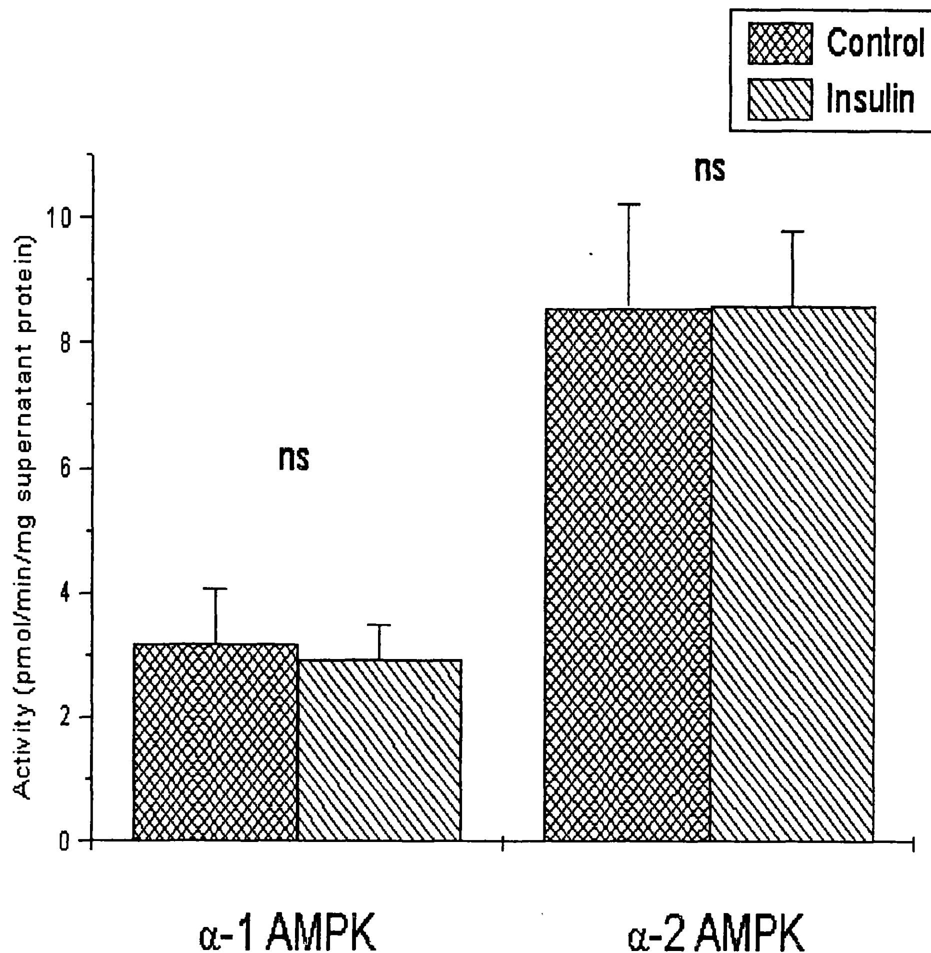
Figure 3.18. Effects of Insulin on AMPK Activity.



Hearts were perfused with 5mM glucose, 2% BSA ± 10nM insulin where indicated. α-1 or α-2 AMPK complexes were immunoprecipitated and assayed in the presence of 200μM AMP.

Values are the mean ± SEM of 6-8 independent perfusions. * indicates $p < 0.05$ compared to control perfusions.

Figure 3.19. Effect of Insulin on AMPK Activity in Palmitate Perfused Hearts.



Hearts were perfused with 5mM glucose, 0.5mM palmitate, 2% BSA ± 10nM insulin. α-1 or α-2 AMPK complexes were immunoprecipitated and assayed in the presence of 200μM AMP.

Values are the mean ± SEM of 6 independent perfusions. ns indicates no significant difference compared to hearts perfused without insulin.

activity in adult rat hearts (Gamble J and Lopaschuk GD, 1997), and AMPK activity is also decreased relative to controls in response to 100μU/ml insulin in the perfused newborn rabbit heart (Makinde AO *et al.*, 1997).

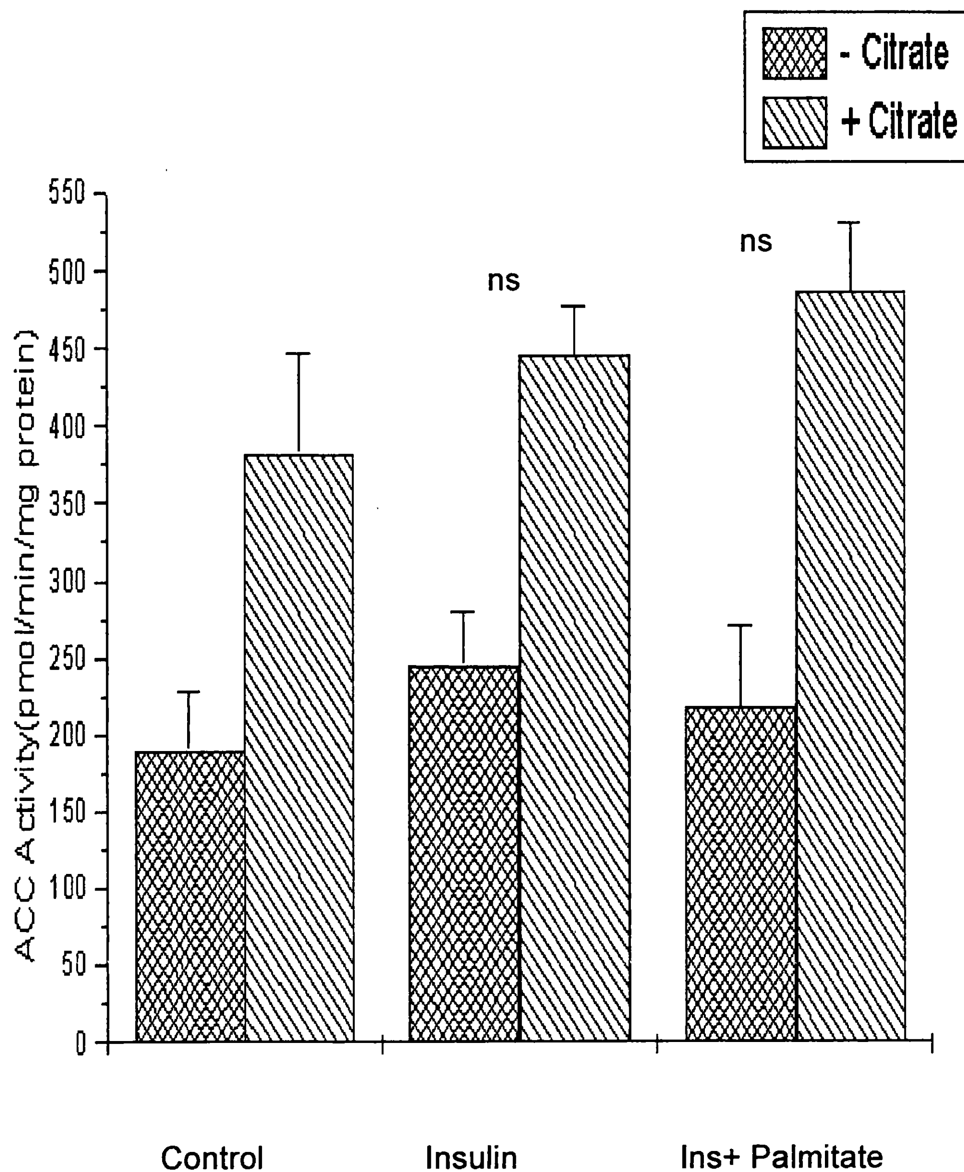
3.2.1.3. Effect of Insulin on ACC Activity.

The effect of perfusion with insulin in the presence or absence of 0.5mM palmitate on ACC activity was determined in 100,000g supernatants as described in section 2.8.2. Despite the expectation of an increase in activity, as can be seen in figure 3.20. no change in total or citrate-dependent ACC activity could be observed in response to insulin treatment.

Insulin treatment has been shown to increase ACC1 activity in adipocytes (Haystead TAJ and Hardie DG, 1986) and hepatoma cells (Witters LA and Kemp BE, 1992). The mechanism by which insulin exerts this effect is uncertain. Insulin treatment of adipose tissue (Halestrap AP and Denton RM, 1974) and hepatocytes (Borthwick AC *et al.*, 1987) increases the amount of ACC in the polymerised form. Insulin has been proposed to activate liver ACC1 by dephosphorylation (Mabrouk GM *et al.*, 1990) and by increased phosphorylation in both adipocytes (Brownsey RW and Denton RM, 1982) and hepatocytes (Holland R and Hardie DG, 1985) at the 'I' site. The relevance of this phosphorylation has been questioned as the insulin effect is not reversed by protein phosphatases and in adipocytes is overcome by okadaic acid.

The effect of insulin treatment on ACC2 activity is not clear. Saha *et al* 1997 showed that the increase in skeletal muscle malonyl-CoA which occurred in response to insulin was not associated with measurable changes in ACC activity. In working rat hearts perfused with palmitate, insulin treatment had no stimulatory effect on citrate-dependent ACC activity (Sakamoto J *et al.*, 2000). One day old rabbit hearts contain carboxylase activity that is increased after perfusion with insulin, the effect being lost in seven day old hearts (Makinde AO *et al.*, 1997). Whether this represents true ACC activity is unclear as all measurements were made in the absence of citrate. In contrast measurable increases in ACC activity have been observed in 6% PEG pellets from hearts perfused with 1000μU/ml insulin compared to control

Figure 3.20. Effects of Insulin on ACC Activity.



Hearts were perfused with 5mM glucose and 2% BSA. Where indicated insulin (10nM) and/or palmitate (0.5mM) were also included. Activity is shown as the mean ± SEM for 5-6 independent preparations. ns indicates not significantly different from controls.

hearts perfused with a lower (100μU/ml) insulin concentration (Gamble J and Lopaschuk GD, 1997).

3.3.2. Adrenaline.

Stimulation of heart tissue with adrenaline is associated with changes in fuel selection. Increased rates of ATP utilisation occur after adrenergic stimulation, with the energy needs of the heart being met by increasing both glucose and fatty acid utilisation (Collins-Nakai RL *et al.*, 1994). Adrenaline and glucagon treatment of adipocytes (Haystead TAJ *et al.*, 1990) and hepatocytes (Sim ATR and Hardie DG, 1988) has been shown to result in increased phosphorylation of ACC at Ser-79, the site phosphorylated in response to AMPK activation.

Experiments were carried out using hearts perfused with 5μM adrenaline in the presence and absence of 0.5mM palmitate to determine whether the increased rates of fatty acid utilisation resulted from a stimulation of AMPK and/or an inhibition of ACC activities.

Perfusions carried out with adrenaline were non-recirculating to ensure a constant fatty acid supply that was unaffected by hormone-stimulated lipolysis. Hearts perfused in this way showed enhanced rates of lipolysis compared to unstimulated hearts as indicated by the efflux of glycerol into the perfusion media. Glycerol release was increased from 0.13 ± 0.03 to 0.81 ± 0.16μmol/min/g dry weight in the absence of palmitate and from 0.10 ± 0.01 to 0.93 ± 0.23μmol/min/g dry weight for hearts perfused with 0.5mM palmitate. These values are the mean ± SEM for 4 (controls) or 5 (plus adrenaline) independent perfusions.

3.3.2.1. Effect of Adrenaline on Cardiac Malonyl-CoA.

Increased rates of palmitate oxidation occur in response to adrenaline and can be correlated with decreased tissue levels of malonyl-CoA. Adrenaline has been observed to overcome the insulin stimulated increase in malonyl-CoA in rat hearts perfused in the absence of palmitate (Awan MM and Saggerson ED, 1993) but this effect is not seen in isolated suspensions of cardiac myocytes (Hamilton C and Saggerson ED, 2000). Increasing cardiac work by stimulating perfused hearts with 1μM epinephrine and increasing the afterload by 40% has also been shown to reduce tissue malonyl-CoA by 33%

which results in a 40% increase in the rate of β-oxidation (Goodwin GW and Taegtmeyer H, 1999).

In this study the effects of 5μM adrenaline on perfused rat hearts was examined in the absence of insulin. This concentration of adrenaline was chosen as it has been shown previously to increase rates of palmitate oxidation in isolated cardiac myocytes incubated with identical concentrations of glucose and palmitate (Awan MM and Saggerson ED, 1993).

Figure 3.21. shows the effect of adrenergic stimulation on the concentration of malonyl-CoA in hearts perfused with glucose as sole substrate and in perfusions containing 0.5mM palmitate. Adrenaline treatment resulted in a slightly decreased malonyl-CoA content compared to controls, which was significant in the absence of exogenous palmitate. No additive effect of adrenaline and palmitate on cardiac malonyl-CoA content was observed (see figure 3.6.).

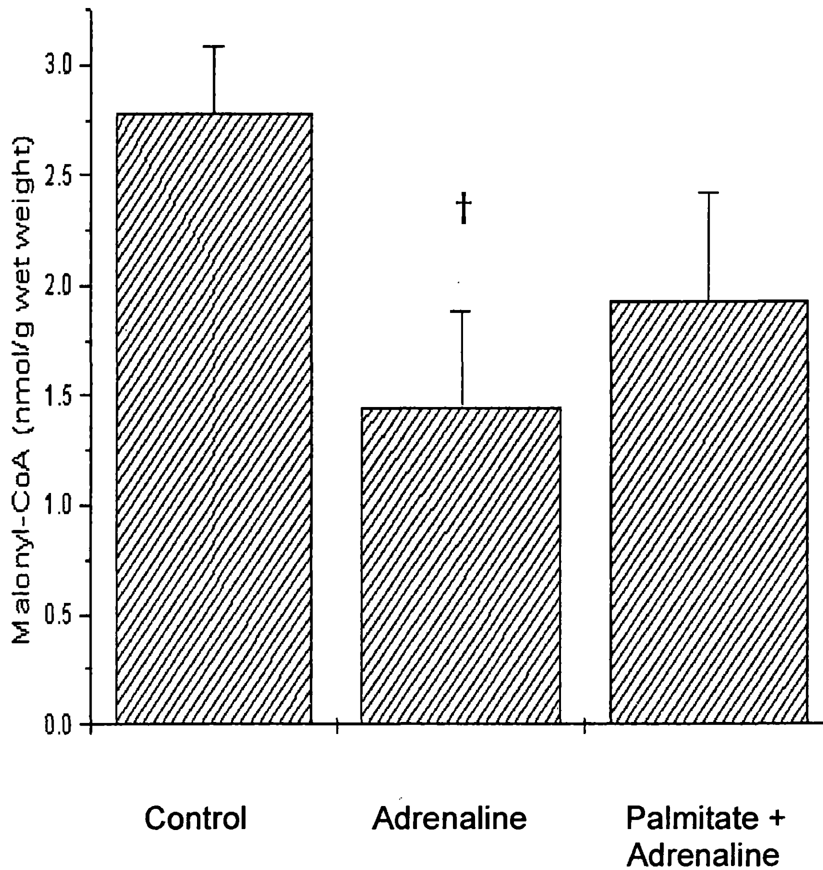
3.3.2.2. Effect of Adrenaline on AMPK Activity.

Figure 3.22. shows the effect of 5μM adrenaline on α-1 AMPK activity in hearts perfused with glucose as the sole substrate. α-1 AMPK activity was significantly ($p<0.01$) increased by adrenaline when AMP was omitted from the assay mixture. Interestingly in hearts perfused with adrenaline, as was the case for palmitate, AMP failed to stimulate kinase activity and measurable changes in activity did not occur under these assay conditions.

Figure 3.23. shows the effect of adrenaline on α-1 AMPK activity in hearts perfused with glucose and 0.5mM palmitate. No significant activation by AMP of the kinase was observed in either heart group and no further increase in kinase activity (above that induced by palmitate) was stimulated by adrenaline.

Figure 3.24. shows the effect of adrenaline on the activity of α-2 AMPK (assayed in the presence of 200μM AMP) in hearts perfused with glucose alone or with 0.5mM palmitate. Despite the expectation that AMPK activity would increase, no activation was observed in hearts perfused with glucose as the sole substrate and interestingly adrenaline significantly ($p<0.01$) overcame the ability of palmitate to stimulate α-2 AMPK activity.

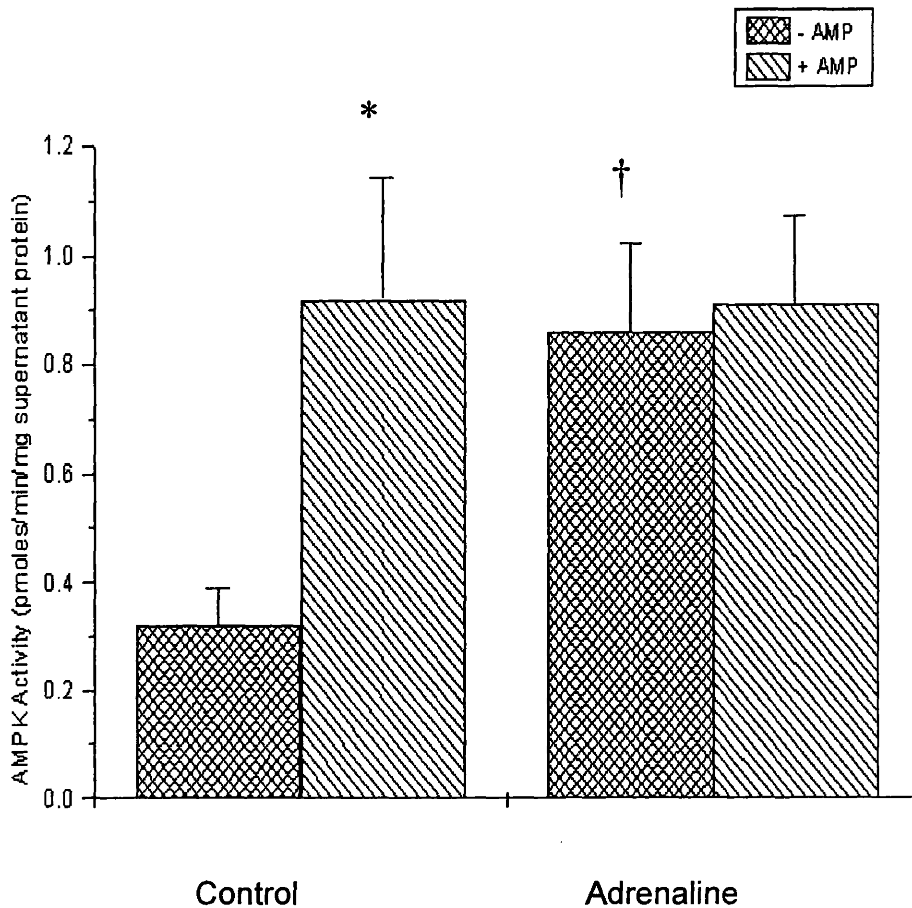
Figure 3.21. Effect of Adrenaline on Malonyl-CoA Concentration.



Hearts were perfused with 5mM glucose and 2% BSA. Where indicated palmitate was included at 0.5mM and adrenaline 5μM.

Values are the mean ± SEM of 4-6 independent perfusions. † indicates $p< 0.05$ for the effect of adrenaline.

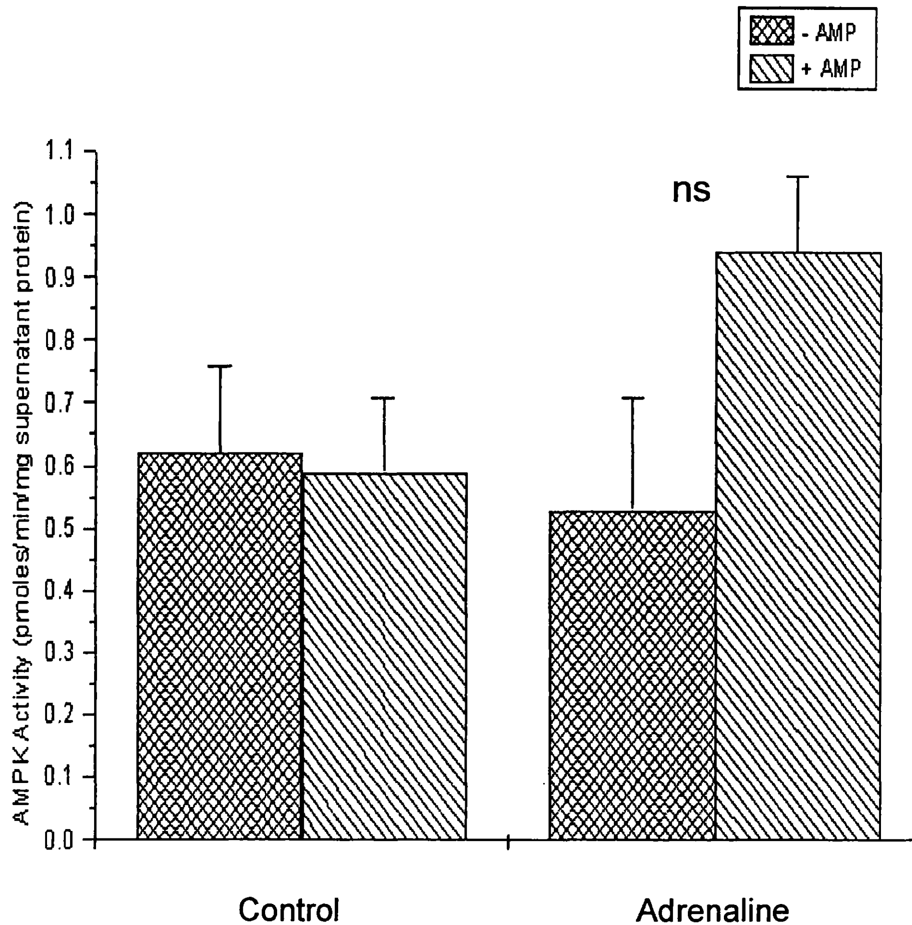
Figure 3.22. Effect of Adrenaline on α-1 AMPK Activity.



Hearts were perfused with 5mM glucose and 2% BSA. Where indicated adrenaline 5μM was included in the perfusion medium. α-1 AMPK complexes were immunoprecipitated and activity measured ± 200μM AMP.

Values are the mean ± SEM of between 4 and 7 independent perfusions. * indicates $p<0.025$ (paired test) for the effect of AMP, † indicates $p< 0.01$ for the effect of adrenaline under the same assay condition.

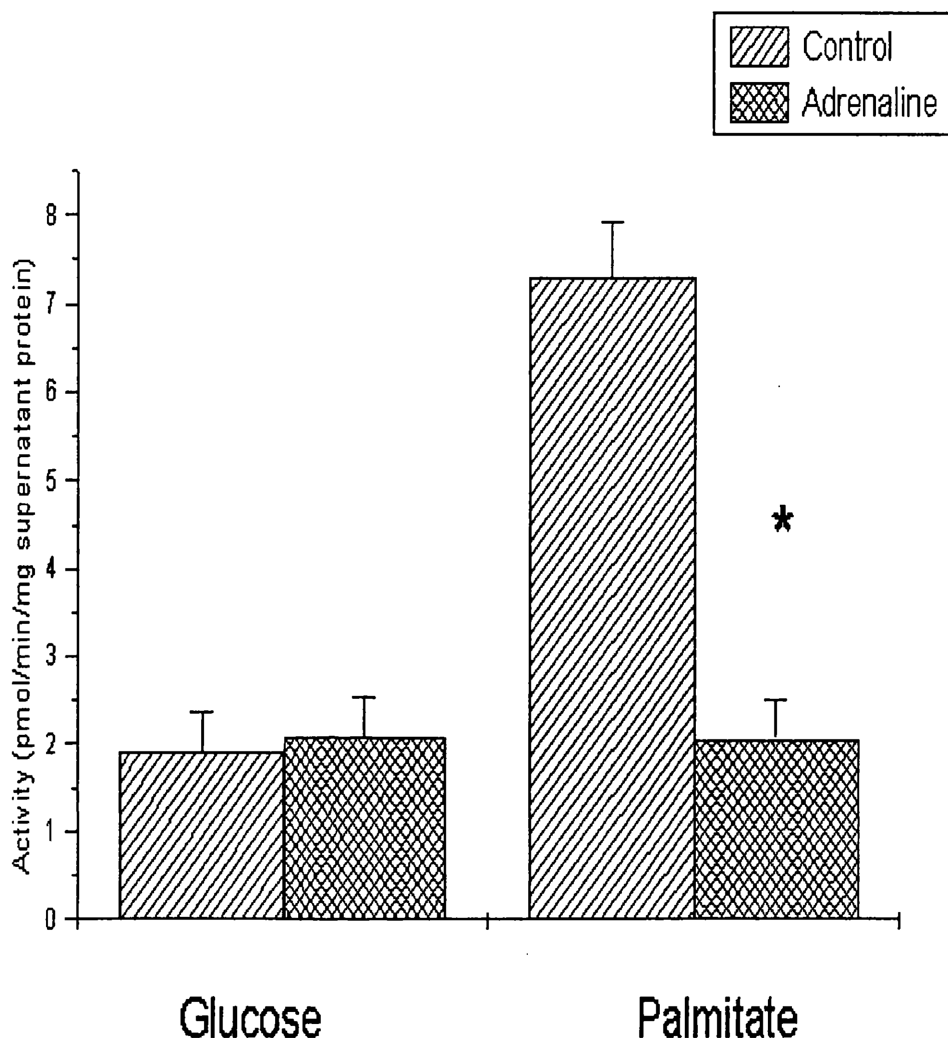
Figure 3.23. Effect of Adrenaline on α-1 AMPK Activity in Palmitate Perfused Hearts.



Hearts were perfused with 5mM glucose, 2% BSA and 0.5mM palmitate. Where indicated adrenaline 5μM was included in the perfusion medium. α-1 AMPK complexes were immunoprecipitated and assayed ± 200μM AMP.

Values are the mean ± SEM for 5 independent perfusions. ns indicates no significant difference compared to perfusions carried out in the absence of adrenaline.

Figure 3.24. Effect of Adrenaline on α-2 AMPK Activity.



Hearts were perfused with 5mM glucose ± 0.5mM palmitate in the presence or absence of 5μM adrenaline. Assays were carried out in the presence of 200μM AMP.

Values are the mean ± SEM of 4 or 5 independent perfusions. * indicates $p < 0.001$ for the effect of adrenaline under the same perfusion condition.

As AMPK containing the α-2 subunit co-purify with cardiac ACC (Dyck JRB *et al.*, 1999), and the inactivation of muscle ACC is reported to be due to this isoform (Vavvas D *et al.*, 1997), the results here do not support the idea that cardiac ACC2 is inactivated by AMPK in response to adrenaline.

3.3.2.3. Effect of Adrenaline on ACC Activity.

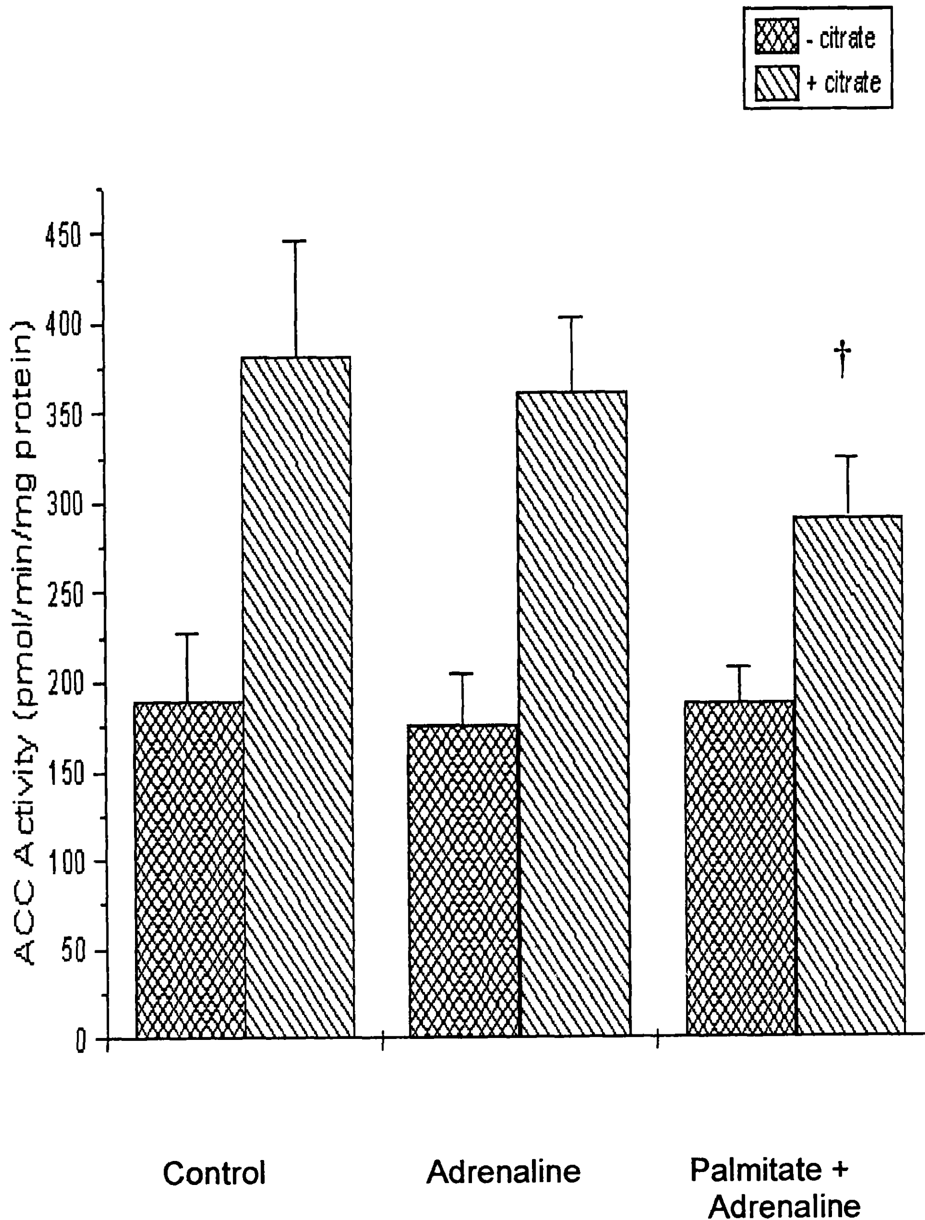
Figure 3.25. shows the result of the inclusion of adrenaline on the activity of ACC in hearts perfused with 5mM glucose in the presence or absence of 0.5mM palmitate. Adrenaline treatment caused a small, but significant ($p<0.05$) decrease in citrate-dependent carboxylase activity when palmitate was included in the perfusion media, but not in hearts perfused with glucose as the sole substrate.

Rat liver ACC2 has been shown to be a better substrate for PKA than ACC1(Winz R *et al.*, 1994). Unlike ACC1 the phosphorylation of heart ACC2 in response to adrenergic stimulation occurs at PKA and not AMPK sites (Boone AN *et al.*, 1999). Rat muscle ACC2 has been shown to be phosphorylated by the catalytic subunit of PKA, without any change in Vmax, Ka for citrate or in its Km for substrates (Winder WW *et al.*, 1997). ACC2 from rat heart is phosphorylated *in vitro* by purified PKA (Dyck JRB *et al.*, 1999) and increased ACC2 phosphorylation occurs in cardiac myocytes after isoprenaline treatment (Boone AN *et al.*, 1999). Whether this phosphorylation results in altered enzyme activity *in vivo* is not clear. In the study by Dyck *et al* the *in vitro* phosphorylation by PKA resulted in a loss of ACC activity. However in the report by Boone *et al*, incubation of cardiac myocytes with isoprenaline did not result in any change in ACC activity that survived purification and assay.

3.4. Cardiac Hypertrophy.

Alterations in cardiac fuel selection occur in response to hypertrophy, with hearts showing a decreased reliance on fatty acids as oxidative fuels. N Bhutta and ED Saggerson (unpublished results) have shown a significant increase in citrate-independent ACC activity in hypertrophied heart tissue. As dephosphorylated ACC has been shown to have substantial citrate-independent activity (Thampy KG and Wakil SJ, 1985), it was of interest to this study to determine whether the decrease in fatty acid utilisation and

Figure 3.25. Effect of Adrenaline on ACC Activity.



Hearts were perfused with 5mM glucose and 2% BSA. Where indicated palmitate 0.5mM and/or adrenaline 5μM were included in the perfusion medium. Assays were carried out ± 10mM citrate.

Values are the mean ± SEM of 5 or 6 independent perfusions. † indicates $p < 0.05$ for the effect of adrenaline.

changes in ACC are due to decreased AMPK activity and result in increases in the tissue level of malonyl-CoA.

Cardiac hypertrophy was induced in male rats (approx. 150g) by ligation of the abdominal aorta above the left renal artery in a surgical procedure performed by N. Bhutta. This ligature causes pressure overload on the heart as well as decreasing the perfusion pressure of the left kidney. This results in vasoconstriction and increased pressure on the heart via a stimulation of the renin-angiotensin system and increases in the levels of angiotensin II, as well as decreased concentrations of the vasodilator bradykinin. Ten weeks after surgery rats were injected with sodium pentobarbitone and hearts removed under anaesthetic and frozen. The degree of hypertrophy was calculated using the ratio of heart weight to tibia length. Sham operated animals in which the aorta was not constricted were used as controls for each experiment.

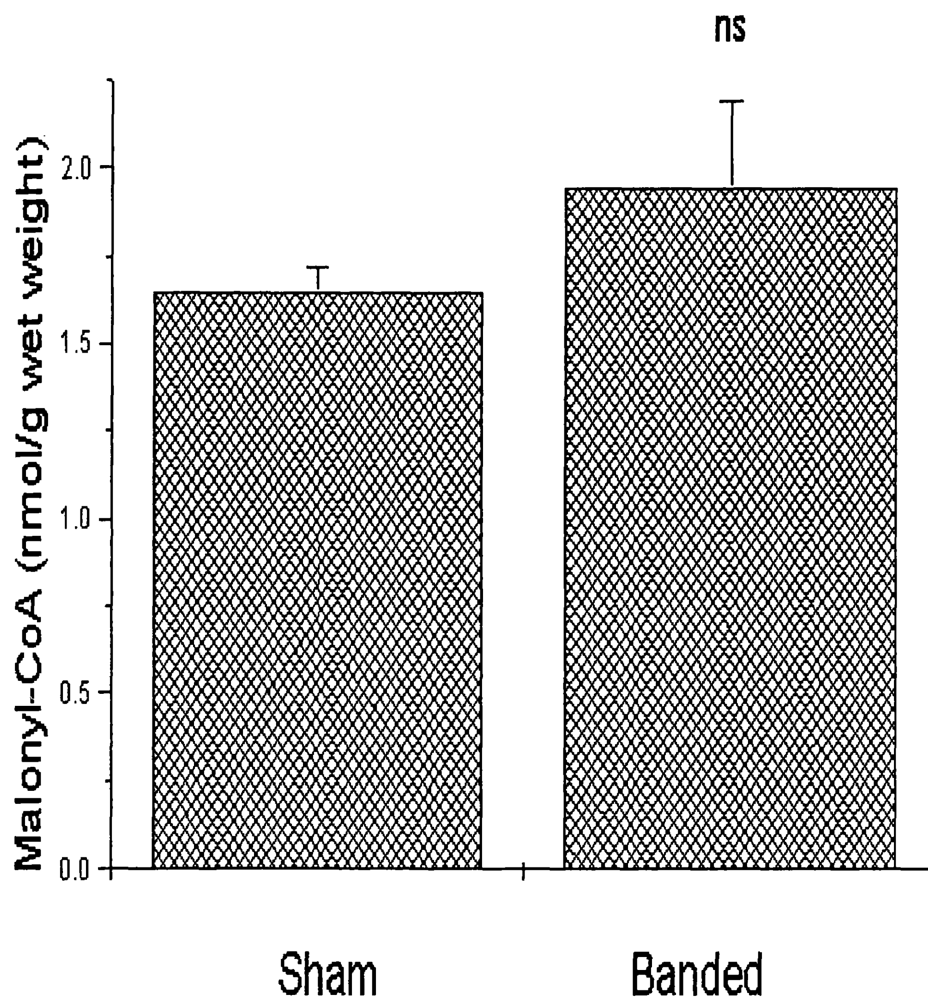
3.4.1. Effect of Hypertrophy on Cardiac Malonyl-CoA.

Figure 3.26 shows the results of experiments to measure malonyl-CoA in hypertrophied heart tissue. No significant difference in malonyl-CoA concentration could be observed between sham and aortic banded hearts.

Although citrate-independent ACC activity was increased in hypertrophied hearts it should be noted that total ACC activity was decreased. It is possible that there is a change in ACC expression in response to hypertrophy, the lower amount of protein being dephosphorylated and hence less dependent on citrate for activation. If this is the case malonyl-CoA levels may be maintained at control levels. In order to clarify this total ACC protein could be measured, as well as the contribution of each of the two ACC isoforms.

It is also possible that malonyl-CoA does not accumulate because of its increased removal by the hypertrophied heart. Potential routes for malonyl-CoA disposal include conversion to acetyl-CoA by MCD, and the synthesis of fatty acid. As the available evidence suggests that MCD expression and activity is decreased in hypertrophy (Young ME *et al.*, 2001) increased conversion to acetyl-CoA seems unlikely. Hypertrophied hearts have an increased demand for phospholipids to support membrane synthesis.

Figure 3.26. Effect of Hypertrophy on Malonyl-CoA Content.



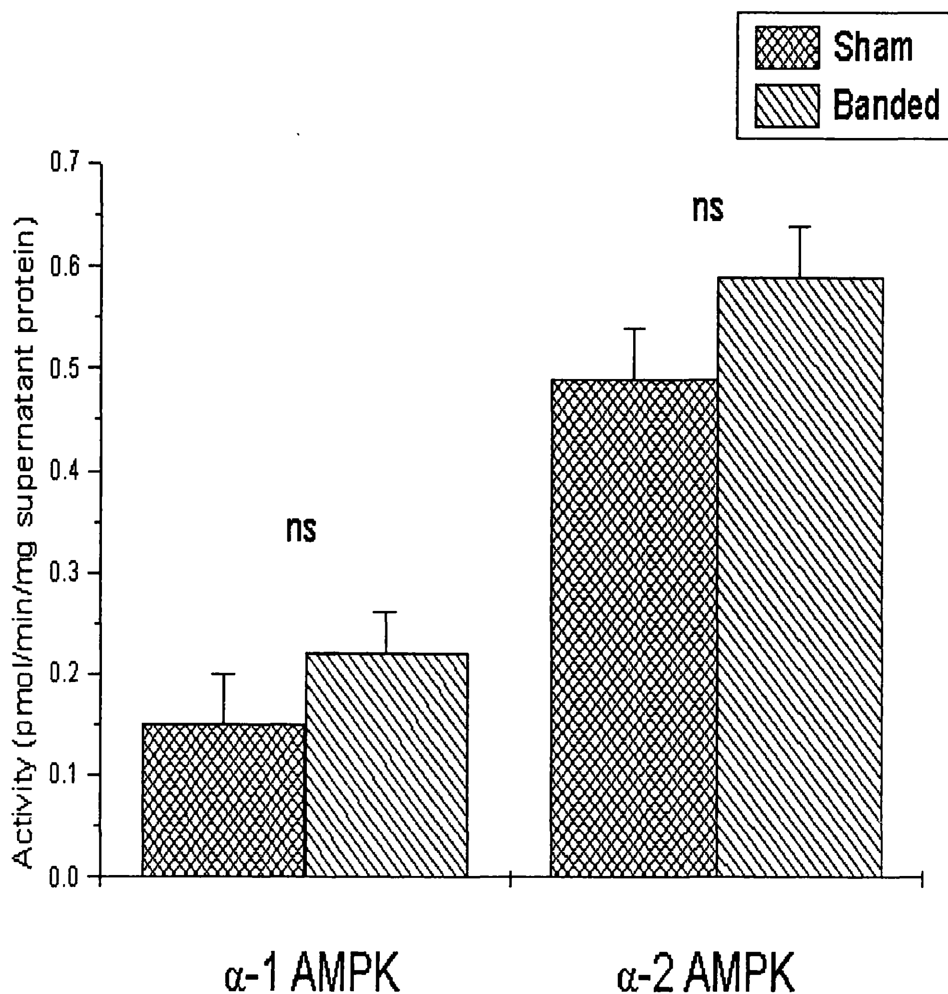
The malonyl-CoA concentration of sham and aortic banded heart tissue was measured after perchloric acid extraction. Values are the mean ± SEM of 8 separate hearts in each group. ns indicates no significant difference versus sham hearts.

Fatty acid synthase activity is low in normal heart tissue, whether this is altered in hypertrophy is not known.

3.4.2. Effects of Hypertrophy on AMPK Activity.

Figure 3.27. shows the effect of cardiac hypertrophy on AMPK activity. No significant difference in either α-1 or α-2 AMPK could be seen in hypertrophied versus sham operated heart tissue. These results imply that the increase in citrate independent ACC activity seen in hypertrophied hearts is not due to alterations in the activity of the AMPK cascade.

Figure 3.27. Effect of Hypertrophy on AMPK Activity.



Heart tissue from sham or aortic banded rats was homogenised and α-1 or α-2 AMPK activity immunoprecipitated. Assays were performed without added AMP.

Values are the mean ± SEM for 5 heart samples in each group. ns indicates no significant difference versus sham hearts.

3.5. General Discussion.

The 'glucose fatty acid cycle' described by Randle *et al* 1963 provided an explanation of the mechanism by which fatty acids are able to inhibit the utilisation of carbohydrate fuels. Inhibitors of CPT1 and oxygen deprivation abolish this effect indicating that it is mediated by the products of β-oxidation. As described in section 1.5.1. increasing fatty acid oxidation inhibits PDH activity as a result of increased mitochondrial NADH and acetyl-CoA, and glycolysis via increased levels of cytosolic citrate. Carbohydrate fuels are also able to inhibit fatty acid utilisation via increases in malonyl-CoA concentration and inhibition of CPT1. As carbohydrate fuels inhibit β-oxidation, and this oxidation is essential to reduce carbohydrate utilisation, a mechanism must be in operation by which fatty acids can signal their own availability and decrease malonyl-CoA concentration.

The main finding of this study is that increasing palmitate supply to isolated perfused hearts results in a significant activation of both α-1 and α-2 AMPK. This stimulation of activity is reversed by PP2C treatment and is associated with a tendency for increased thr-172 phosphorylation. Whether palmitate stimulates an upstream AMPKK or inhibits the action of protein phosphatases remains to be elucidated. The activation of AMPK by palmitate is associated with increased phosphorylation of ACC at the AMPK site. A role for AMPK in the palmitate-stimulated decrease in malonyl-CoA concentration is implied by this observation, although any changes in ACC activity occurring *in vivo* did not survive the extraction and assay conditions utilised in this study.

As some α-2 AMPK is localised in the nucleus (Salt I.P. *et al.*, 1998) and AMPK may affect gene transcription (da Silva Xavier G *et al.*, 2000; Hubert A *et al.*, 2000; Foretz M *et al.*, 1998; Leclerc I *et al.*, 1998) the activation of α-2 AMPK by palmitate measured in this study may also provide a mechanism by which fatty acids could regulate gene expression via a pathway independent of peroxisome proliferator-activated receptors.

No change in cardiac adenine nucleotide concentration occurred after perfusion with palmitate, and the mechanism by which palmitate regulates AMPK is unclear at present. One study on the relationship between AMPK activity and the *in vivo* AMP concentration, as measured by N.M.R. has

shown half-maximal activation at an AMP concentration of 1.8μM, despite high (6.8-11.7mM) concentrations of ATP (Frederich M and Balschi JA, 2002). This observation led the authors to conclude that *in vivo* either ATP antagonism of AMPK activity is reduced or other factors besides AMP are responsible for activating the kinase. In yeast the SNF1 kinase is activated by glucose removal, a situation associated with increases in the AMP/ATP ratio, however SNF1 is not activated *in vitro* by AMP (Wilson WA *et al.*, 1996). In view of the results presented here and other reports of AMPK activation without changes in adenine nucleotides (Beauloye C *et al.*, 2001; Minokoshi Y. *et al.*, 2002) further work is needed to identify alternative regulators of the AMPK cascade.

Perfusion of hearts with 10nM insulin resulted in an increased cardiac malonyl-CoA content, consistent with previous reports (Awan MM and Saggerson ED, 1993; Hamilton C and Saggerson ED, 2000) and observations that insulin decreases the contribution of palmitate derived acetyl-CoA to the citric acid cycle (Sakamoto J *et al.*, 2000). Insulin treatment resulted in an inhibition of AMPK activity, and this effect, as was the increase in malonyl-CoA, was overcome by palmitate. Previous reports have shown similar effects of insulin treatment on AMPK activity. Insulin decreased AMPK activity in hearts perfused with 5mM glucose (Beauloye C *et al.*, 2001), and no effect of the hormone on kinase activity is seen in hearts perfused with 0.4 or 1.2mM palmitate (Sakamoto J *et al.*, 2000). AMPK activity can however be decreased in newborn rabbit hearts perfused with fatty acid (Makinde AO *et al.*, 1997) and in adult rat hearts by a ten-fold increase in insulin concentration (Gamble J and Lopaschuk GD, 1997). The ability of palmitate to override the insulin-stimulated increase in malonyl-CoA and inhibition of AMPK is also interesting with regard to the role of fatty acids in the development of insulin resistance. Elevation of plasma fatty acid concentration causes insulin resistance in human skeletal muscle, an effect associated with decreased insulin-stimulated glucose transport and decreased IRS-1 associated PI 3-kinase activity (Dresner A *et al.*, 1999). Although again malonyl-CoA concentration was shown to reflect changes in AMPK activity no link could be made with ACC activity which was unchanged by insulin treatment.

The mechanism by which insulin exerts its inhibitory effect on AMPK activity remains to be elucidated. The phosphatidylinositol-3-kinase inhibitor wortmannin is able to overcome the insulin-stimulated decrease in AMPK activity (Beauloye C *et al.*, 2001), whilst the p70 ribosomal S6 kinase inhibitor rapamycin was without effect. Further work needs to be carried out to determine whether glucose is necessary for insulin to exert its effect on AMPK as the hormone has been shown to be unable to increase malonyl-CoA concentrations in cardiac myocytes incubated in its absence (Hamilton C and Saggerson ED, 2000).

Perfusion of hearts with 5μM adrenaline resulted in interesting effects on AMPK activity, with the two isoforms showing different responses to the hormone. As fatty acid utilisation is increased on adrenergic stimulation it was expected that kinase activity, being sensitive to fatty acid, would increase after adrenaline treatment. The activity of α-1 AMPK was stimulated by adrenaline after perfusion with glucose as the sole substrate, the activity matching that seen after perfusion with palmitate. No activation above that due to palmitate was observed when hearts perfused with 0.5mM palmitate were stimulated by adrenaline. In hearts perfused with 0.5mM palmitate α-2 AMPK activity was significantly decreased after adrenergic stimulation, with rates of SAMS peptide phosphorylation similar to hearts perfused with glucose alone. Adrenaline had no effect on α-2 AMPK activity in the absence of fatty acid. These effects of adrenaline on AMPK activity are unlikely to be due to PKA phosphorylation. PKA does not directly affect AMPK activity or its phosphorylation by AMPKK (Davies SP *et al.*, 1989).

These combined results show that α-1 AMPK is activated under conditions that increase fatty acid utilisation. The activity of complexes containing the α-1 subunit increases with increasing [palmitate] and is decreased in response to insulin when rates of fatty acid oxidation are reduced. In hearts stimulated with adrenaline the oxidation of endogenous fatty acid is increased with no change in exogenous fatty acid utilisation (Goodwin GW *et al.*, 1998; Kreisberg RA, 1966). The activation of α-1 AMPK seen as a result of adrenaline treatment relative to controls could therefore be explained by the increased availability of fatty acid released from myocyte TAG stores. No stimulation of α-1 AMPK activity could be attributed to adrenaline in palmitate

perfused hearts. This implies that the kinase was fully activated in response to exogenous fatty acid in both palmitate and adrenaline-stimulated hearts.

Although α-2 AMPK was activated in response to increasing [palmitate] and decreased by insulin, its sensitivity to fatty acid could be questioned by the failure of adrenaline to stimulate its activity in control hearts and by its ability to overcome the activation due to palmitate. Although TAG lipolysis is accelerated the increased energy needs of the heart after adrenergic stimulation are met primarily by increased glycolysis and glucose oxidation (Collins-Nakai RL *et al.*, 1994). Therefore the activity of AMPK containing α-2 subunits seems to be reduced under conditions where glucose utilisation is increased, i.e. in response to perfusion with insulin and adrenaline and in the absence of exogenous fatty acid. It is also possible that adrenaline is able to antagonise the activation of α-2 AMPK by palmitate via α-adrenergic signalling pathways. Future work utilising specific agonists and antagonists of the α- and β- adrenoreceptors would be necessary to determine the contribution of each pathway to the effect of adrenaline on AMPK activity.

Pyruvate has been shown to decrease rates of glucose transport in cardiomyocytes (Fischer Y *et al.*, 1997) and to decrease glycolysis in the perfused rat heart via inhibition of PFK1 (Garland P.B. *et al.*, 1963). If the activity of α-2 AMPK is inhibited at high glycolytic rates, pyruvate would be expected to increase its activity. In this study no alteration in the activity of α-2 AMPK was observed when hearts were perfused with 0.2 or 0.5mM pyruvate (in the presence of 5mM glucose). The sensitivity of AMPK to glucose has been demonstrated in other cell types. Yeast SNF1 kinase becomes activated when glucose is removed from the growth medium. Pancreatic β-cell AMPK has been shown to be inactivated in response to increased glucose supply (Salt I.P. *et al.*, 1998) (da Silva Xavier G *et al.*, 2000). In contrast increasing glucose from 5 to 25mM does not alter AMPK activity in rat hepatocytes (Foretz M *et al.*, 1998). Malonyl-CoA levels in rat cardiomyocytes have been shown to increase as incubation [glucose] is raised (Hamilton C and Saggerson ED, 2000), therefore the effect of altering glucose supply on heart AMPK activity warrants further study.

Whether the two AMPK isoforms have distinct substrates is not clear. It has been reported that the liver α-1 isoform, but not α-2, is able to phosphorylate

both liver ACC isoforms (Michell B.J. *et al.*, 1996). Decreases in ACC activity seen during contraction in skeletal muscle are paralleled by increases in the activity of α-2 AMPK (Vavvas D *et al.*, 1997). The co-purification of α-2 AMPK with ACC has led to the suggestion that this isoform is responsible for the regulation of ACC activity in heart tissue (Dyck JRB *et al.*, 1999). If this is shown to be the case the changes in α-2 kinase activity measured here could reflect alterations in malonyl-CoA, with the exception of adrenaline perfusions where it is possible that phosphorylation of ACC by PKA is the major controlling factor. The ability of adrenaline to overcome the palmitate-induced stimulation of α-2 AMPK is interesting with regard to the mechanism of HSL regulation. AMPK phosphorylation is able to prevent the activation of HSL by PKA, therefore a decrease in AMPK activity may be necessary to ensure provision of fatty acid from TAG stores after adrenergic stimulation. However the AMPK isoform responsible for the phosphorylation of HSL has not been determined.

No significant change in the activity of ACC that would explain the altered tissue contents of malonyl-CoA was seen in this study, except for a small decrease due to adrenaline in palmitate perfused hearts. ACC was almost totally dephosphorylated at the AMPK site in control hearts compared to those perfused with palmitate and the reason why this did not translate to changes in carboxylase activity is unclear. It is possible that the failure to measure alterations in ACC activity was due to a problem with the method employed. In order to clarify this further experiments could be carried out to assay ACC under conditions where activity has previously been shown to change. Kudo *et al,* 1995 observed a decrease in total carboxylase activity in hearts reperfused after a period of ischemia, however whether this change reflects true ACC activity is questionable as citrate-dependent ACC activity was actually increased in the Kudo study. To effectively evaluate the reliability of the ACC assay method tissue from an alternative organ could be used. Hepatic ACC is known to be phosphorylated and inactivated in response to starvation and the feeding of high fat diets. By employing the tissue preparation and assay methods utilised in this study to replicate this effect would allow clarification of whether the results presented here are due to problems in the methodology or a true lack of effect.

Purified rat muscle ACC is inhibited after *in vitro* phosphorylation by AMPK but not by PKA (Winder WW *et al.*, 1997). Purified rat heart ACC2 is phosphorylated and inactivated *in vitro* by PKA and AMPK (Dyck JRB *et al.*, 1999). Although ACC2 is phosphorylated in cardiac myocytes exposed to isoprenaline no change in carboxylase activity that survived extraction and assay could be detected (Boone AN *et al.*, 1999).

It is possible that alterations in ACC phosphorylation alone are insufficient to produce changes in activity that survive purification and assay. The extended N-terminal sequence of ACC2 has been proposed to regulate the association of the enzyme with the mitochondrial membrane (Ha J *et al.*, 1996). A later study has shown ACC2 to be localised on the mitochondrial membrane in a variety of cells (Abu-Elheiga L *et al.*, 2000). Whether the phosphorylation state of the enzyme can regulate membrane association is not known. A protein regulator able to activate ACC has been isolated from rat liver (Quayle KA *et al.*, 1993). This activation is not due to changes in ACC phosphorylation and appears to be due to a specific protein-protein interaction. It is therefore possible that if such a regulator exists in heart tissue, phosphorylation of ACC2 may regulate this association.

The observation that ACC2 has a lower affinity for acetyl-CoA than ACC1 has led to suggestions that the activity of this isoform is dependent on substrate supply. Malonyl-CoA levels increase in hearts perfused with the PDH activator DCA without alterations in ACC activity (Saddik M *et al.*, 1993). The increase in malonyl-CoA was proposed to be due to increased acetyl-CoA efflux from the mitochondria, increasing substrate for the ACC reaction. This theory was based on an observation by the same research group that supplementing hearts with carnitine stimulated glucose oxidation and inhibited fatty acid oxidation (Broderick TL *et al.*, 1992). This effect was proposed to be due to the stimulation of carnitine acetyltransferase and acetyltranslocase activities, reactions of which acetyl-CoA derived from pyruvate but not β-oxidation is a substrate (Lysiak W *et al.*, 1986). This process could effectively remove inhibition of PDH by acetyl-CoA, increase malonyl-CoA and inhibit β-oxidation, although malonyl-CoA levels were not measured in the study by Broderick *et al.* However, for acetyl-CoA derived from acetylcarnitine to be available as a substrate for ACC, the presence of a cytosolic carnitine

acetyltransferase is required. Evidence for the existence of such activity is lacking (Edwards YH *et al.*, 1974; Abbas AS *et al.*, 1998). Also of note is a study by Abdel Aleem in 1996 in which cardiac myocytes supplemented with carnitine showed increased palmitate oxidation rates with no change in rates of glucose oxidation (Abdel-aleem S *et al.*, 1996).

The activity of ACC may also be influenced by the cytosolic concentration of citrate. Citrate may activate ACC allosterically or increase provision of the acetyl-CoA substrate via ATP-citrate lyase. In skeletal muscle the increases in malonyl-CoA in response to glucose and glucose plus insulin are associated with increases in tissue citrate plus malate, an antiporter for mitochondrial citrate efflux (Saha AK *et al.*, 1997). In the same study it was shown that these increases in malonyl-CoA could be overcome by inhibition of ATP-citrate lyase, results that imply a role for citrate derived acetyl-CoA as the major ACC substrate. Citrate concentration increases in hearts perfused with insulin (Lawson JWR and Uyeda K, 1987) and decreases with adrenaline (Vincent G *et al.*, 2000) corresponding with the changes observed in malonyl-CoA in this study. However malonyl-CoA levels decrease in the heart in response to perfusion with palmitate, a condition previously shown to increase citrate levels (Garland P.B. *et al.*, 1963). If the supply of acetyl-CoA from citrate is a factor in the regulation of cardiac malonyl-CoA concentration, the control of ATP-citrate lyase activity, particularly in response to fatty acid may also be important.

The involvement of MCD in the regulation of cardiac malonyl-CoA concentration cannot be ruled out. Although a mitochondrial enzyme, MCD has been shown to have overt activity in isolated heart mitochondria (Hamilton C and Saggerson ED, 2000) and may therefore be accessible to cytosolic malonyl-CoA. MCD is activated in rat hearts subjected to increased workload, a situation associated with decreased malonyl-CoA and increased rates of fatty acid oxidation (Goodwin GW and Taegtmeyer H, 1999). MCD activity is also decreased as a chronic effect in the hearts of rats fed high fat diets (Young ME *et al.*, 2001).

The mechanism by which MCD activity is regulated is not clear. Reports on the effect of AMPK activation on MCD activity are contradictory. Increases in MCD activity due to contraction in skeletal muscle parallel the activation of

AMPK (Saha AK *et al.*, 2000), and MCD activity was stimulated after incubation of muscles with AICAR. However changes in MCD activity do not always follow AMPK activation. No alteration of MCD activity is seen in exercising skeletal muscle or in pancreatic islet cells exposed to low glucose or AICAR (Habinowski SA *et al.*, 2001), or in the ischaemic rat heart (Dyck JRB *et al.*, 1998). Recombinant MCD or MCD immunoprecipitated from heart or skeletal muscle is not phosphorylated in response to incubation with AMPK *in vitro* (Habinowski SA *et al.*, 2001).

3.6. Future Work.

In order to further understanding of the mechanisms by which fatty acids and hormones are able to regulate malonyl-CoA concentration and rates of β-oxidation the following experiments should be performed:

1. Measurements of MCD activity in response to varying perfusion [palmitate].
2. Measurements of AMPK activity in response to palmitate after treatment of hearts/myocytes with the fatty acyl-CoA synthase inhibitor triacsin C would enable the contribution of this reaction to the increase in AMPK activity after palmitate exposure to be determined.
3. Measurements of AMPK activity also need to be carried out after exposure to different chain length fatty acids to determine whether the activation of AMPK is specific to palmitate.
4. Measurements of AMPK activity after perfusion with varying [glucose] to determine whether increased glucose utilisation is inhibitory.
5. Perfusion of hearts with insulin in the absence of glucose followed by measurement of AMPK to determine whether glucose is necessary for the effects of the hormone.
6. Measurement of AMPK activity after perfusion with isoprenaline and phenylephrine would allow the pathway by which the adrenaline effect on AMPK is mediated to be elucidated.

Chapter Four

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