The Effect of Glucose on Cardiac AMP-Activated Protein Kinase

by

Ikhlass Tabidi

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Department of structural and Molecular Biology

University College London
Gower Street
London WC1E6BT

Declaration

I, Ikhlass Tabidi, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that it has been indicated in the thesis

Signed……………………………………….................Date…………

Abstract

AMP-activated protein kinase (AMPK) serves as an energy-sensing protein that is activated by a variety of metabolic stresses. Recent studies suggest that AMPK is also regulated by hormones and by nutrients such as glucose and fatty acids. In skeletal muscle it was previously shown that AMPK activity was decreased with increasing glucose concentration. In the present study both the activity and the Threonine-172 phosphorylation of AMPK in incubated rat ventricular cardiac myocytes were found to be decreased by increasing glucose in the presence and absence of palmitate. Glucose also caused a decrease in the AMPK-driven phosphorylation of acetyl-CoA carboxylase. Measurements of the myocyte contents of ATP, ADP, AMP and glycogen showed that the effect of glucose on AMPK activity could not be secondary to changes in the levels of these metabolites. The decrease in AMPK activity with glucose was additive to and distinct from the effect of insulin which is mediated through protein kinase B (PKB). Increasing glucose concentration had no effect on the phosphorylation of Threonine-308 and Serine-473 in PKB. AICAR, a pharmacological activator of AMPK, had no effect on the ability of glucose to inactivate AMPK. The myocyte content of the pentose phosphate pathway (PPP) metabolite xylulose 5-phosphate (Xu5P), a known allosteric activator of PP2A, was increased with increasing glucose concentration such that AMPK activity was inversely related to Xu5P content. The glucose 6-phosphate dehydrogenase inhibitor diehydroepiandeosterone (DHA) and thiamine, the precursor of the coenzyme for transketolase, both increased AMPK activity whereas the NADPH oxidant phenazine methosulphate (PMS) decreased AMPK activity. DHA and PMS respectively decreased and increased flux through the PPP. The findings suggest that inactivation of AMPK by glucose may be mediated by the activity of the PPP which sets the level of Xu5P. Two other findings were also made during the course of this project. First, the activity of phosphofructokinase-2 (PFK-2) in the perfused heart was previously shown to be activated through phosphorylation by AMPK. It was therefore expected that increasing glucose

concentration would decrease myocyte PFK-2 activity. However PFK-2 activity was found to be increased by glucose. Second, the phosphorylation of Threonine-308 in PKB in response to insulin was appreciably increased in the presence of AICAR suggesting that there may be crosstalk between AMPK and the insulin signalling pathway at the level of PKB.

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Abbreviations

ACC	acetyl-CoA carboxylase
ACOD	acyl-CoA oxidase
ALD	aldolase
ADP	adenosine 5'-diphosphate
AKAP	A-kinase anchoring ptrotein
AMP	adenosine 5'-monophosphate
AMPK	AMP-activated protein kinase
AMPKK	AMP-activated protein kinase kinase
AICAR	5-aminoimidazole-4-carboxamide ribonucleoside
aPKC	atypical protein kinase C
ASC	Association with SNF1 Complex
ASK1	apoptosis signal-regulated kinase 1
ATP	adenosine 5'-triphosphate
BCA	bicinchoninic acid
BSA	bovine serum albumin
C	carboxyl (C)-terminus
C	catalytic subunit
CAMK	calcium/calmodulin-dependent protein kinase
CAMKK	calcium/calmodulin-dependent protein kinase kinase
cAMP	cyclic adenosine monophosphate
CBS	cystathione-ß-synthase
CHO	Chinese hamster ovary
cPKC	conventional protein kinase C
Cr	creatine
CrP	phosphocreatine

DAG	diacylglycerol
DNP	dinitrophenol
DTT	dithiothreitol
DHAP	dihydroxyacetone phosphate
DHA	dehydroepiandrosterone
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
eEF	eukaryotic elongation factor
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
FABPpm	plasma membrane associated fatty acid binding protein
FAT	fatty acid translocase
GAP	GTPase activator proteins
F-6-P	fructose-6-phosphate
F-2,6-P$_2$	fructose2,6-bisphosphate
F-1,6-P	fructose 1,6-bisphosphate
FBPase	fructose bisphosphatase
Glu-6-P	glucose-6-phosphate
GAP	glyceraldehyde-3-phosphate
GBD	glycogen binding domain
G3-P	glyceraldehyde3-phosphate
GHD	glycerol-3-phosphate dehydrogenase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
G6PD	glucose- 6-phosphate dehydrogenase
Glut	glucose transporter
GPCR	G protein coupled receptor

GRK	G protein coupled receptor kinase
GS	glycogen synthase
GSK3	glycogen synthase kinase 3
GFAT	glutamine fructose 6-phosphate-amidotransferase
GTP	guanosine 5'-triphosphate
HK	hexokinase
Hepes	4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HPLC	high performance liquid chromatography
HRP	horseradish peroxidise
HSL	hormone sensitive lipase
HUVEC	human umbilical vein endothelial cell
IGF-1	insulin-like growth factor-1
IP_3	inositol-1,4,5-trisphosphate
IPG	Inisitol phosphoglycan
JAK1	Janus kinase 1
JNK	c-Jun N-terminal kinase
KHB	Krebs Henseleit bicarbonate buffer
LCFA	long-chain fatty acid
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
MEF2	myocyte enhancer factor 2
MEHA	3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline
MEK	mitogen-activated protein kinase/extracellular signal-regulated kinase kinase
MEKK	mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase
MCD	malonyl-CoA decarboxylase

MKKK	mitogen-activted protein kinase kinase kinase
MO25α	mouse protein 25 α
mTOR	mammalian target of rapamycin
Myr	myristoylation
MUK	MAPK upstream kinase
N	amino (NH_2)-terminus
NAD^+	nicotinamide adenine dinucleotide (oxidised)
NADH	nicotinamide adenine dinucleotide (reduced)
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NFAT	nuclear factor of activated T lymphocytes
NHERFP-1/2	Na^+-H^+ exchanger regulatory factor proteins-1/2
nPKC	novel protein kinase C
NSF	N-ethylmaleimide-sensitive factor
PA	phosphatidic acid
PP2A	phosphoprotein phosphatase 2A
PAK	p21-activated kinase
PC	phosphatidylcholine
PCA	perchloric acid
PAP	phosphatidic acid phosphohydrolase
PDH	pyruvate dehydrogenase
PDK1	phosphoinositide-dependent protein kinase 1
PEG	polyethylene glycol
PMS	Phenazine methosulphate
PFK2	fructose-6-phosphate-2-kinase
PP_I-PFK	Fructose-6-phosphate phosphotransferase

PH	pleckstrin homology
PI3K	phosphoinositide-3-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PKD	protein kinase D
PKI	protein kinase inhibitor
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PM	plasma membrane
PMA	phorbol-12-myristate-13-acetate
PMSF	phenylmethanesulfonyl fluoride
PP	protein phosphatase
Pi	inorganic phosphate
PS	phosphatidylserine
PtdIns	phosphatidylinositol
$PtdIns(3,4,5)P_3$	phosphatidylinositol-3,4,5-trisphosphate
$PtdIns(3,4)P_2$	phosphatidylinositol-3,4-bisphosphate
$PtdIns(3)P$	phosphatidylinositol-3-phosphate
$PtdIns(4,5)P_2$	phosphatidylinositol-4,5-bisphosphate
PVDF	polyvinyldene difluoride
PYK2	protein tyrosine kinase 2
PPP	pentose phosphate pathway
R	regulatory subunit
RACK	receptor for Activated C-kinase
RICK	receptors for Inactive C-kinase
R5-P	ribose 5-phosphate

Rictor	rapamycin-insensitive companion of mTOR
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoreses
SH	Src homology
siRNA	small interfering RNA
SNF1	Sucrose non-fermenting 1
SPRK	Src homology 3 domain-containing proline-rich protein kinase
SR	sarcoplasmic reticulum
STRADα	STE20-related adaptorα
TAK1	transforming growth factor-ß-activated kinase
TBS	Tris buffered saline
Tris	tris(hydroxymethyl)-aminomethane
TSC2	tuberous sclerosis complex 2
TPP	thiamine pyrophosphate
TPI	triosephosphate isomerise
X5-P	xylulose 5-phosphate
UDPGlcNac	UDP-N-acetylglucosamine
UCP1	uncoupling protein 1
ZMP	5-aminoimidazole-4-carboxamide ribonucleoside monophosphate

Chapter-1- General Introduction

General Introduction

The heart can utilize a variety of carbohydrate and lipid fuels to generate energy in the form of adenosine triphosphate (ATP) for contractile function. The rate of oxidation of these fuels depends on the demands of the heart. The increase in work rate increases ATP demand which leads to increases in oxidative phosphorylation and citric acid cycle activity. Under normal conditions, the myocardium takes its fuels from the bloodstream so that the energy stores of glycogen and triacylglycerol (TAG) are preserved.

The oxidation of fatty acids is a very effective source of energy compared to glucose. Normally, it provides 60-70% of the energy requirement of the myocardium. It has been well established that fatty acids inhibit the utilization of carbohydrate in the heart through sequences of regulatory mechanisms known as the glucose/fatty acid cycle. A major regulator of fatty acid and glucose metabolism during stress is the AMP-activated protein kinase. It has also become increasingly clear that AMPK can be regulated by nutrients and hormones. Under a variety of pathological conditions, it has been proved that high levels of circulating fatty acids markedly decrease glucose utlization by the heart. The strict control of fuel selection in the heart is essential for efficient contractile functioning. The purpose of this study is to examine the effects of carbohydrates and insulin on AMPK activity in cardiac myocytes.

1.2 Cardiac Energy Metabolism

The heart can produce energy from a wide range of substrates including dietary fatty acids and carbohydrates as well as from various molecules produced during their metabolism, such as lactate, pyruvate and ketone bodies. Carbohydrates are not the preferred myocardial fuel under normal conditions. Decreased perfusion during myocardial ischemia limits the delivery of oxygen and nutrients and decreases oxidative metabolism. Under these conditions, increased glycolytic ATP production becomes a critical energy source. Several mechanisms have been proposed to account for the increases in glucose uptake and glycolysis during myocardial ischemia. These include: α-adrenergic mechanisms, p38 activation, and more recently, the stimulation of AMP-activated protein kinase (AMPK).

1.3 AMPK

AMPK is a member of a metabolite-sensing protein kinase family that is found in all eukaryotes (Hardie *et al.;* 1998). AMPK has been proposed to act as a cellular fuel sensor, which controls metabolism to balance energy supply and demand. A decreased cellular energy supply leads to an AMPK-mediated adaptation to allow cellular survival. In doing so, AMPK acts as a cellular "fuel gauge" (Hardie *et al.;* 1998)..

During stress, such as nutrient deprivation, AMPK will inhibit energy-consuming pathways while it promotes energy producing pathways, e.g., fatty acid oxidation and glycolysis. This simple principle is not only operative at the cellular level, because AMPK also governs whole body energy metabolism by the regulation of food intake in the hypothalamus (Xue and Kahn 2006).

1.3.1 The role of AMPK
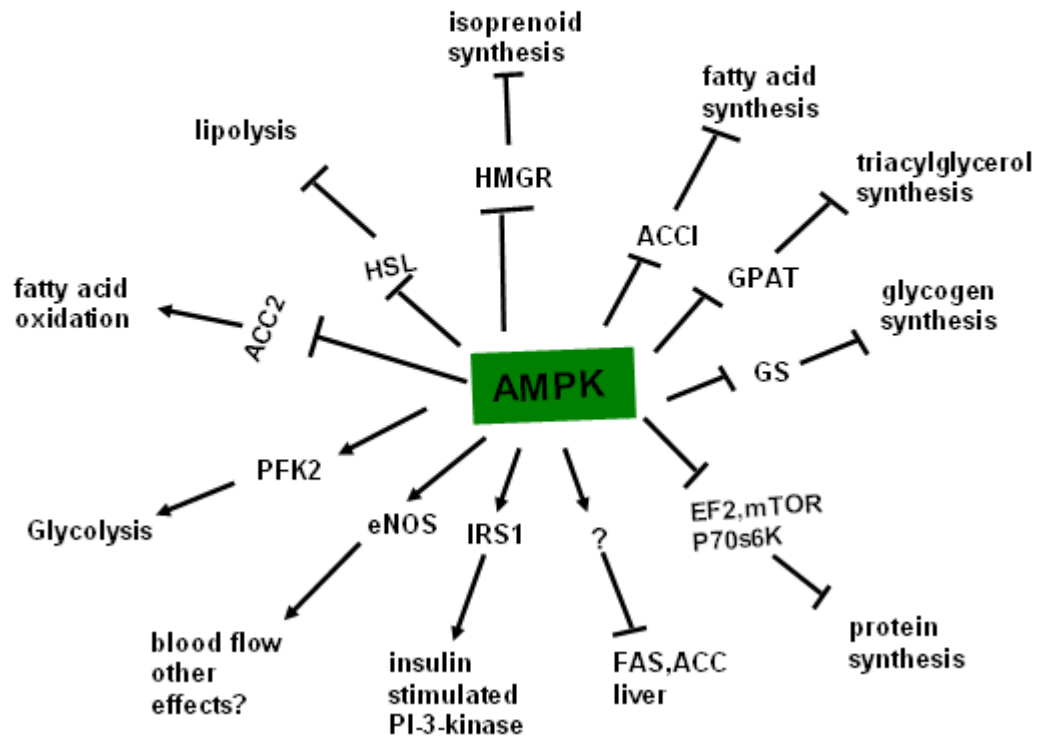
Figure 1.1: **The role of AMP-activated protein kinase (AMPK) on energy substrate utilization:** AMPK activates energy-generating pathways such as glycolysis by increasing glucose uptake, phosphofructokinase-2 activity and fatty acid oxidation. The later occurs via a number of mechanisms such as the increased uptake of fatty acids from the circulation through the recruitment of lipoprotein lipase to the endocardia surface as well as promoting the translocation and/or retention of the fatty acid transporter (CD36) at the plasma membrane. In addition, AMPK may inhibit energy-consuming pathways, such as protein and glycogen synthesis (Hardie *et al.;* 2003).

1.3.2. AMPK Structure

Mammalian AMPK is a trimeric enzyme comprising a catalytic α subunit (63 kDa) and non-catalytic β and γ subunits (**Figure 1.2**). Multiple isoforms of each mammalian enzyme subunit exist (α1, α2, β1, β2, γ 1–γ 3), each encoded by a different gene. The β subunits have a calculated molecular mass of 30 kDa, but migrate on SDS/PAGE with apparent masses of 38 kDa (β1) and 34 kDa (β2), whereas the three γ isoforms have molecular masses of 37 kDa (γ 1), 63 kDa (γ 2) and 55 kDa (γ 3).

The catalytic α subunit (Carling *et al* 1989) transfers ATP to serine or threonine residues in the consensus sequence (Φ(X,β)XXS/T*XXXΦ, where Φ and β indicate hydrophobic and basic residues, respectively; X indicates any amino acid; the order of the amino acids within the parenthesis is not important) (Weekes *et al* 1993; Dale *et al* 1994; Salt *et al* 1998). However, a structural modelling of the interaction between the catalytic domain of AMPK and the region around Ser79 (AMPK phosphorylation site) of ACC1 has suggested that residues over a much longer region than the above recognition motif are important for the interaction (Scott *et al* 2002). There are two isoforms of the α subunit; α1 (548 residues) encoded by the gene on chromosome 5p11 (Stapleton *et al* 1997) and α2 (552 residues) encoded by the gene on chromosome 1p31 (Beri *et al* 1994) (Stapleton *et al* 1996). Both isoforms migrate on SDS-PAGE at approximately 63 kDa and are activated by AMP and by the phosphorylation of Thr172 by AMPKK (Stapleton *et al* 1996). They show high sequence similarity at the N-terminal catalytic domain but less similarity at the C-terminal region (Stapleton *et al* 1996). Truncation studieshaveshown that the C-terminal region (313-473) of the α subunit is required for association with the β and γ subunits (Figure 1.3) (Crute *et al* 1998; Iseli *et al* 2005). A truncated α1 subunit lacking the C-terminal region (α1 (312)) is constitutively active and the presence of an autoinhibitory domain has been suggested in the region between residues 312-392 (Crute *et al* 1998). The α1 mRNA and protein is generally expressed but α2 mRNA and protein is most abundant in heart, skeletal muscle and liver (Stapleton *et al* 1996). In terms of the activity, α2 AMPK activity is predominant in the heart and skeletal muscle, while α1 AMPK activity is predominant in lung, kidney, testis, brain and pancreas (Cheung *et al*

2000). Both α1 and α2 AMPK contribute equally in the liver (Cheung *et al* 2000). In the heart, α1 AMPK contributes to about 30% of the total AMPK activity (Cheung *et al* 2000).

The α1 and α2 isoforms display a number of differences. The α2 activity is stimulated by AMP to a greater extent than α1 *in vitro* (Salt *et al* 1998). This effect was seen for both the allosteric effect of AMP and for the stimulation of the Thr172 phosphorylation by an upstream kinase. This difference in the sensitivity to AMP may explain the greater activation of α2 AMPK than α1 AMPK during cardiac ischemia (Li *et al* 2006) and the isoform-specific AMPK activation during skeletal muscle contraction. Low or moderate skeletal muscle contraction has been generally found to selectively activate α2 AMPK while α1 activation is only seen during high intensity contraction (Vavvas *et al* 1997; Stephens *et al* 2002; Wojtaszewski *et al* 2000; Chen *et al* 2000), although a recent study by Toyoda *et al* has shown a selective activation of α1 AMPK bylow intensity contraction in the epitrochlearis muscle (Toyoda *et al* 2006). Both isoforms are deactivated similarly by PP2C but α2 is deactivated by PP2A to a much greater extent than α1 (Salt *et al* 1998). α1 and α2 AMPK may also have distinct physiological functions. It has been shown only α2 AMPK co-purifies with heart ACC (Dyck *et al* 1999) suggesting a role for this isoform in the regulation of cardiac ACC. α2 AMPK has also been suggested to be the isoform involved in the regulation of glucose homeostasis (Jorgensen *et al* 2004). A selective activation of α2 has been also observed in skeletal muscle in response to leptin. Both α1 and α2 AMPK are found in the cytosol but α2 AMPK is also found in the nucleus in INS-1 cells and CCL13 cells (Salt *et al* 1998). This localisation is not dependent on the activation state of α2 or association with the β and γ subunits (Salt *et al* 1998).

The β-subunit composition of the AMPK heterotrimer complex does not have enzymatic activity. There is no evidence of a preferential association of the two β subunit isoforms with different α isoforms in *vitro* (Thornton *et al.*; 1998). The β subunit binds both the α and γ subunits and is thought to act as a scaffold protein. Both isoforms are widely distributed. The liver expresses

mainly β-1 and a small amount of β-2, while the opposite pattern is seen in skeletal muscle (Thornton *et al.*; 1998). The heart expresses both β isoforms but β-2 is slightly more abundant than β-1 (Thornton *et al.*; 1998). The yeast homologues of the β subunit contain domains termed KIS and ASC which are involved in the association with Snf1p and Snf4p, respectively (Yang *et al.*; 1994). The internal region of the β subunits which overlaps with the putative KIS domain has been identified as the glycogen binding domain (GBD), that is closely related to the isoamylase domain found in glycogen and starch branching enzyme (Polenkhina *et al.*; 2003). The β subunit is myristoylated at its N terminus (Thornton *et al.*; 1998). The myristorylation is involved in the membrane association of AMPK (Mitchelhill *et al.;* 1997). The removal of the myristoylation has also been shown to increase AMPK activity through a mechanism that is independent of a change in the α-Thr 172 phosphorylation (Arden *et al.*; 2001).

The γ-subunit carries four cystathione-β-synthase motifs (CBS), organized into two pairs (CBS1/2) and (CBS3/4) called Bateman domains, which are proposed to bind AMP and ATP (Adams *et al.*; 2004). Mutation in these domains makes AMPK insensitive to AMP (Hamilton *et al.*; 2001). Moreover, several naturally occurring mutations in the human γ-2 subunit, which are associated with the development of the Wolf Parkinson-White syndrome, are found within the CBS domains, and these mutations have been shown to decrease the sensitivity of AMPK to AMP (Daniel and Carling; 2002). γ-1 and γ-2 isoforms are expressed widely whereas γ-3 mRNA is found only in skeletal muscle (Cheung *et al.*; 2000). The heart expresses both γ-1, which is the predominant form, and γ-2 Lie *et al.*; 2006) **(Figure 1.2).**
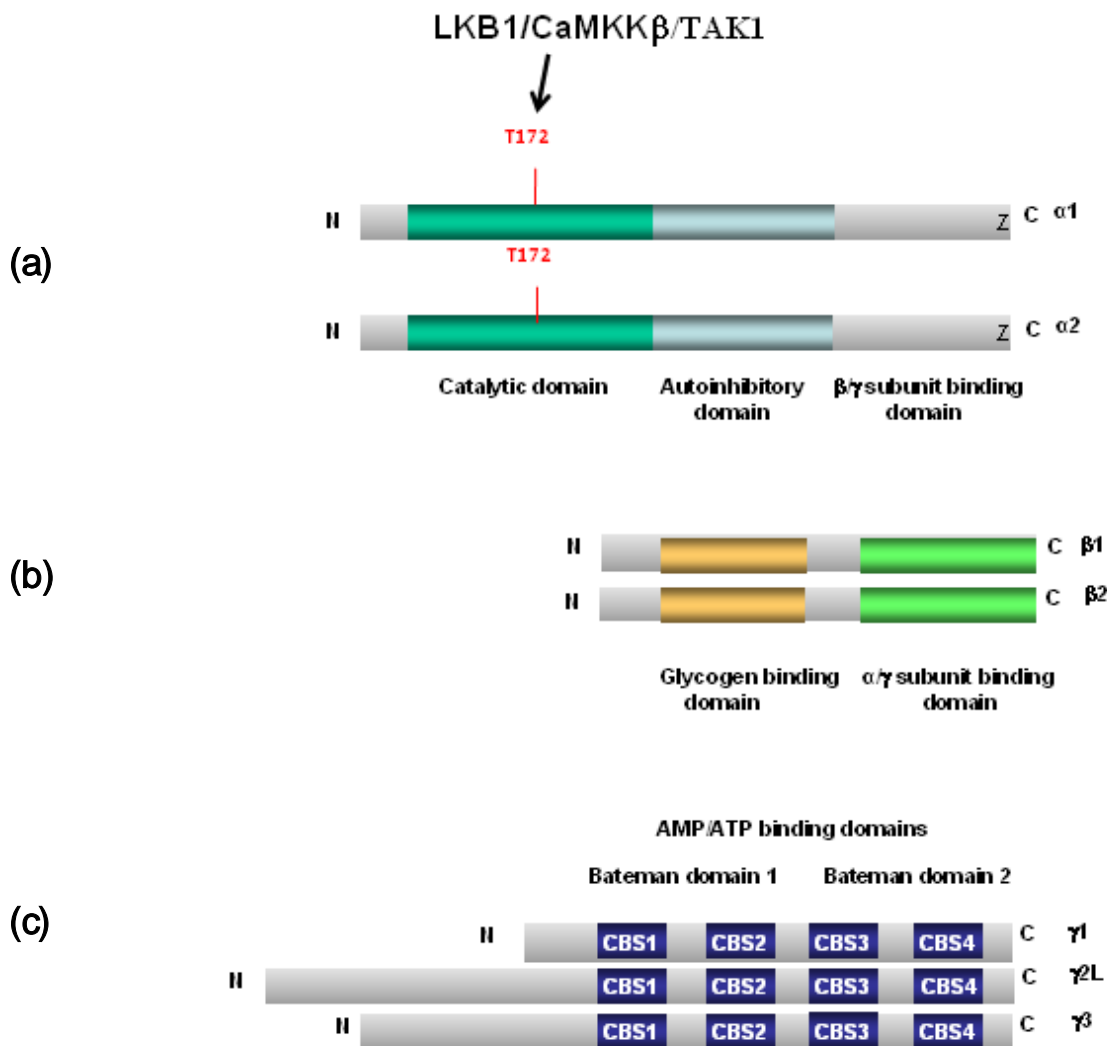
Figure 1. 2: Structure of AMPK. The mammalian (a) AMPK (α1 and α 2), (b) AMPK (β 1 and β2) and (c) AMPK (γ1, γ2γ 3). The α -subunits contain the Thr 172 residue that must be phosphorylated by upstream kinases for activity and an autoinhibitory sequence domain that inhibits the activity of the kinase domain. The C-terminal domain is required for binding the β - and γ-subunits. The β -subunits contains a central glycogen-binding domain and a C-terminal domain that is required for binding the α - and γ- subunits. The three γ-subunit isoforms have variable N-terminal domains and four conserved cystathionine beta-synthase motifs (CBS1-4). The CBS motifs act in pairs to form two Bateman domains that bind the AMP or ATP.

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1.3.3 **General features of the regulation of AMPK**

AMPK is activated by phosphorylation of the α-Thr172 of the α subunit by AMPK kinases (LKB1, TAK1 and CaMKKβ). The mutation of α-Thr172 to alanine completely abolishes the activation of AMPK by partially purified AMPKK (Stein *et al.;* 2000). The identity of the upstream kinase(s) that phosphorylate α-Thr172 was elusive for a long time. Earlier studies on AMPKK mostly used a partially purified AMPKK fraction from rat liver. Recently, three yeast AMPKK homologes have been identified (Pak1p, and Tos3p and Elm1p) which can phosphorylate the residue equivalent to α-Thr172 in the activation loop of Snf1p (Hong *et al* 2003.; Sutherland *et al.;* 2003). Subsequently, LKB1 in mammals, which has a catalytic domain related to these three yeast kinases, has been shown to phosphorylate α-Thr172 and activate AMPK *in vitro* (Hong *et al.;* 2003; Hawley *et al.;* 2003.; Woods *et al.;* 2003; Shaw *et al.;* 2005). LKB1 is known as a serine/threonine kinase, which has a tumour suppressor role. The mutation of LKB1 causes Peutz-Jeghers syndrome (Boudeau *et al.;* 2003). LKB1 (~ 50kDa) purified from rat liver is associated with two accessory proteins; STRADα (~ 45/48 kDa) and MO25α (~ 40kDa) (Hawley *et al.;* 2003). The heterotrimeric complex of LKB1 is required for its full activity towards AMPK (Hawley *et al.;* 2003). LKB1 seems equally to activate AMPK complexes containing different subunit isoforms (Woods *et al.;* 2003). The role of LKB1 as an AMPKK *in vivo* is supported by several observations. First, an anti-LKB1 antibody could remove most of the AMPKK activity from the partially purified liver AMPKK fraction (Hawley *et al.;* 2003; Woods *et al.;* 2003). Second, in cultured cell lines, the inhibition of LKB1 through the expression of catalytically inactive LKB1 inhibited the increase in α-Thr172 phosphorylation induced by AICAR and H_2O_2 (Woods *et al.;* 2003; Shaw *et al.;* 2003). Third, in HeLa cells, which lack endogenous LKB1, and in MEF cells from a LKB1 knock out mouse, AICAR or H_2O_2 failed to increase α-Thr172 phosphorylation (Hawley *et al.;* 2003; Shaw *et al.;* 2003). Lastly, in transgenic mice, the deletion of LKB1 in the liver or

skeletal muscle greatly decreased the basal α-Thr172 phosphorylation and almost completely abolished the increase in α-Thr172 phosphorylation caused by metformin, AICAR, or skeletal muscle contraction (Shaw *et al.;* 2005; Sakamoto *et al.;* 2005). The heart from the transgenic mice lacking the cardiac expression of LKB1 also showed an almost complete abolition of the basal activity of α-2 AMPK and a moderate reduction in the activity of α-1 AMPK, indicating a significant role of LKB1 in cardiac AMPK regulation (Sakamoto *et al.;* 2006).

Despite the lack of LKB1, a small amount of α-Thr172 phosphorylation could still be detected in the HeLa cells and mouse embryo fibroblasts (MEFs) cells from LKB1 knock out mice (Hawley *et al.;* 2003). This was not due to the non-phospho specific binding of the anti-Thr172 antibody, as it was almost completely abolished by incubation with PP2A (Hawley *et al.;* 2005).

A Ca^{2+} ionophore was shown to increase α-Thr172 phosphorylation and AMPK activation in HeLa cells. These observations prompted the search for an AMPKK other than LKB1. The AMPKK activity towards recombinant AMPK present in an HeLa cell lysate could be completely abolished by STO-609, an inhibitor of CAMKKα and β (Hurley *et al.;* 2005). The effect of a Ca^{2+} inophore to activate AMPK in the HeLa cells was also inhibited by STO-609 or by small interfering RNA (siRNA) against CAMKK α or β, although the inhibitory effect of siRNA against CAMKKβ was much greater than that for CAMKKα (Hurley *et al.;* 2005; Hawley *et al.;* 2005; Woods *et al.;* 2005). Both CAMKKα and β can phosphorylate α-Thr172 and activate recombinant AMPK *in vitro*, but CAMKKβ activates AMPKK more readily than CAMKKα (Hawley *et a.;l* 2005; Woods *et al.*; 2005). These observations strongly suggest that, in addition to LKB1, CAMKKβ and possibly CAMKKα also act as AMPKKs *in vivo*. In brain slices, the activation of AMPK by depolarisation or by an increase in Ca^{2+} induced by increasing K^+ concentration has been shown to be inhibited by STO-609, suggesting a role for CAMKKs in the regulation of AMPK in this organ (Hawley *et al.;* 2005). However, although CAMKKα and β are

highly expressed in the brain, they are poorly expressed in other tissues (Anderson *et al.;* 1998). The heart has

been reported to express CAMKKβ but not CAMKKα (Allard *et al.;* 2006). The role of CAMKKβ in the regulation of cardiac AMPK remains to be determined.

There still remains the possibility of the existence of AMPKKs different from LKB1 or CaMKKs. The possibility of a yet unidentified AMPKK that is activated during ischemia has been reported in the heart (Aharejos *et al.;* 2005) . Suzuki and co-workers have demonstrated that, in several cultured cell lines, including the human pancreatic cancer cell line (PANC-1), HeLa and human fibroblast cell lines, AMPK α-Thr172 phosphorylation is increased by incubation with growth factors, such as IGF-1 (Suzuki *et al.;* 2004). The tyrosine phosphorylation of ATM (ataxia telangiectasia mutated) appears to be involved in this effect, as the activation of AMPK was blocked by genistein or antisense mRNA for ATM (Suzuki *et al.;* 2004). The ATM immunoprecipitated from the IGF-1-stimulated cells phosphorylated the AMPK α catalytic domain *in vitro*, suggesting that ATM may be directly upstream of AMPK (Suzuki *et al*.; 2004).

In addition to α-Thr172, AMPK is known to be phosphorylated at several sites on the α and β subunits.The α subunit has been shown to be phosphorylated at two other sites, α-Thr258, and at Ser485 (α1) or Ser 491 (α2) (Woods *et al*.; 2003). The mutation of α-Thr258 or Ser485/491 to aspartic acid to mimic phosphorylation did not affect the kinase activity or sensitivity of AMPK to AMP (Woods *et al.;* 2003). Using constitutively active and catalytically inactive mutants of AMPK, these sites were shown to be phosphorylated by both autophosphorylation and by a partially purified AMPKK (Woods *et al.;* 2003). However, the upstream kinases that phosphorylate α-Thr258 and Ser485/491 were thought to be different from those that phosphorylate α-Thr172 because the sequences surrounding the α-Thr258 and Ser485/491 were similar to each other but different from the sequence surrounding the α-Thr172 (Woods *et al.;* 2003). Unlike α-Thr172, the Ser485 phosphorylation was also resistant to incubation with PP2C,

PP1 or PP2A (Woods *et al.;* 2003). Recently, Horman *et al* have confirmed that α-Thr258 and Ser485 are autophosphorylated but they are not phosphorylated by LKB1 (Horman *et al.;* 2006). Moreover, PKB has been shown to phosphorylate recombinant α1β1γ1 and α2β1γ1 AMPK *in vitro* at Ser485/491. The phosphorylation of Ser485 did not directly affect the kinase activity, but the *in vitro* preincubation of AMPK with PKB and phosphorylation of Ser485 decreased the α-Thr172 phosphorylation and the activation of AMPK by LKB1, suggesting a hierarchical mechanism whereby the prior phosphorylation of Ser485 inhibits the subsequent phosphorylation at α-Thr172 (Horman *et al.;* 2006).

The β1 subunit has been reported to be phosphorylated at Ser24, Ser25, Ser96, Ser101, Ser108, and Ser182 (Mitchelhill *et al.;* 1997; Woods *et al.;* 2003; Chen *et al.;* 1999). The phosphorylations of Ser24/25 are mutually exclusive (Mitchelhill *et al.;* 1997). The only reported phosphorylation on the β-2 subunit is Ser182 (Chen *et al.;* 1999). Phosphorylation of these residues may regulate the AMPK activity, cellular localisation, or interaction with substrate. Warden *et al.* showed that mutation of Ser108 to alanine decreased AMPK activity, while mutation of Ser24/25 or Ser182 did not affect the activity but increased the nuclear localisation of the enzyme (Warden *et al.;* 2001). Ser108 is within the GBD, and mutating it to glutamic acid has been shown partially to inhibit the association of GBD with glycogen (Polekhina *et al* 2003). Most of the β phosphorylation sites identified are autophosphorylation sites (Mitchelhill *et al.;* 1997; Woods *et al.;* 2003). However Ser182 and some of the other sites are also likely to be phosphorylated by separate kinases (Mitchelhill *et al.;* 1997). The catalytically inactive mutant of AMPK has been reported to be phosphorylated at the β subunit by a partially purified AMPKK (Mitchelhill *et al.;* 1997; Stein *et al.;* 2000), although this was not observed by other investigators (Woods *et al.;* 2003). This discrepancy is likely to be due to the difference in the composition of the partially purified AMPKK used in these studies. Recently, the incubation of recombinant AMPK with PKB has been shown to increase phosphate incorporation into the β-1 but not the β-2

subunit (Horman *et al.;* 2006). The significance of PKB phosphorylation on the β subunit in the regulation of AMPK remains to be determined.

 In vitro, AMP activates AMPK by a direct allosteric mechanism and by a decrease in the dephosphorylation of AMPK at α-Thr172 by protein phosphatase (Carling *et al*.; 2007)

This phosphorylation is the key event which accounts for most of the activation of AMPK by AMP. Recent studies in cultured cells and in skeletal muscle lacking LKB1 expression have suggested that LKB1 is required for the phosphorylation of AMPK by treatments that increase the AMP/ATP ratio (Carling *et al*.; 2007). However, it has been shown that AMP does not directly activate LKB1, although the phosphorylation of AMPK by LKB1 is stimulated in the presence of AMP (Hawley *et al.;* 2003; Woods *et al.;* 2003; Shaw *et al.;* 2003). The activity of LKB1 purified from cultured cells or from skeletal muscle treated with agents that increase AMP/ATP is also no different from that in untreated cells (Woods *et al.;* 2003; Shaw *et al.;* 2003; Sakamoto *et al.;* 2004). LKB1 is known to be phosphorylated at up to eight residues (Boudeau *et al.;* 2003), and LKB1 immunoprecipitated from a liver AMPKK fraction exists as several species, which seem to represent different phosphorylation states of LKB1 (Woods *et al.;* 2003). However, none of the phosphorylations of LKB1 appear to regulate LKB1 catalytic activity (Sapkota *et al.;* 2001 and 2002). Consistent with this, the preincubation of LKB1 with PP1, PP2A or PP2C does not affect the phosphorylation of AMPK by LKB1 (Woods *et al.;* 2003), although LKB1 appears to be the predominant AMPKK in the liver.

Whether CAMKK is involved in the phosphorylation of AMPK in response to an increase in AMP/ATP is not clear. CAMKKα and β are activated by Ca^{2+}/calmodulin but they are not directly activated by AMP (Hawley *et al.;* 2005; Woods *et al.;* 2005). This observation alone does not rule out the possibility that AMP facilitates the phosphorylation of Thr172 by CAMKKβ which has been reported to have some activity in the absence of stimulation by Ca^{2+}/calmodulin

(Anderson *et al.;* 1998). Several other observations suggest that CAMKKs specifically mediate the phosphorylation of Thr172 in response to Ca^{2+}. In HeLa cells, which lack LKB1, AMPK is not activated by AICAR or H_2O_2 but is activated by a Ca^{2+} ionophore (Hurley *et al.;* 2005; Hawley *et al.;* 2005). In brain slices, STO-609 inhibited the activation of AMPK by Ca^{2+} but it did not affect the activation of AMPK by Phenformin which increased the cellular AMP/ATP (Hawley *et al.;* 2005). In contrast to these studies, Woods and co-workers showed that the Thr172 phsophorylation could be increased by H_2O_2 in HeLa cells and in LKB1-deficient MEF cells (Woods *et al.;* 2005). Moreover, the transfection of HeLa cells with siRNA against CAMKKβ decreased the basal AMPK activity and both H_2O_2 and ionomycin stimulated AMPK activity (Woods *et al.;* 2005). They also showed that in NIH3T3 cells, which express both LKB1 and CAMKKs, the activation of AMPK by ionomycin, AICAR and H_2O_2 were only partially inhibited by STO-609, whereas in the LKB1-deficient MEF cells, their effects were completely inhibited by the inhibitor, suggesting that Ca^{2+} and AMP activate AMPK via both LKB1 and CAMKKβ (Woods *et al.;* 2005). Offering a possible explanation for the discrepancy between this study and those by Hurley *et al.;* 2005 and Hawley and co-worker described above, these authors suggested that although LKB1 and CAMKKβ are likely to be responsible for the activation of AMPK by AMP and Ca^{2+} signalling, respectively, the distinction is likely to be blurred *in vivo* because an increase in Ca^{2+} is often associated with an increase in AMP and vice versa, possibly due to the action of the **Ca^{2+}** pump (Woods *et al.;* 2005). Recently, (Sanders *et al*.; 2007), using a recombinant preparation of AMPK and LKB1, failed to detect any stimulation of α Thr172 phosphorylation by AMP under any condition. Another group published results using recombinant AMPK in which they are unable to detect an effect of AMP on α Thr172 phosphorylation by LKB1 or CaMKK*β* (Suter *et al.;* 2006) (**Figure 1.3**). However, the authors were unable to account for the apparent discrepancy between their results and those of previous studies. The only explanation for this discrepancy is that the partially purified preparation of AMPK/LKB1 used in previous studies examining AMP stimulation could contain an additional

factor that mediates the effect of AMP on α Thr172 phosphorylation. AMPK has been reported to be inhibited *in vitro* by physiological concentrations of phosphocreatine (PCr) (5-40mM), and this effect has been shown to be antagonised by creatine (Cr) (Ponticos *et al.;* 1998). The regulation of AMPK activity by Cr/CrP is consistent with the energy sensing role of AMPK. The effect of Cr/CrP on AMPK is not

affected by the presence of AMP, suggesting that Cr/PCr binds to a site distinct from the AMP binding site (Ponticos *et al.;* 1998). Whether CrP/Cr affects the phosphorylation of Thr172 by AMPKKs remained unknown. Recently, (Taylor *et al.;* 2006) showed that physiological concentrations of CrP (5-50 mM) did not affect the activation of recombinant α1β1γ1 or α2β2γ2 by the recombinant LKB1 complex or the activation of AMPK purified from the liver by recombinant LKB1. Moreover, the activity of α1β1γ1 or α2β2γ2 were not inhibited by CrP, and Cr did not activate α2β2γ2 or α2β2γ2 by AMPK complex recombinant LKB1 (Taylor *et al.;* 2005). However, they did observe an inhibitory effect of CrP on crude AMPK purified from rat liver and skeletal muscle by polyethylene glycerol PEG) precipitation. However, this effect was not specific for CrP (glucose-6-phosphate also inhibited crude AMPK), and the inhibitory effect by CrP or glucose-6-phosphate was not seen in the purer preparation of AMPK obtained by immunoprecipitation. This suggests that the inhibitory effect of CrP seen in the crude AMPK preparation and possibly that observed by Ponticos and co-workers may be an artefact due to contaminating enzymes, such as creatine kinase and phosphatases, that act on CrP or other small phosphomolecules and possibly perturb the concentrations of free phosphate, ATP or AMP (Taylor *et al.;* 2005). Furthermore a recent study has been unable to detect a direct allosteric effect on AMPK activity or on its upstream kinase by PCr or Cr in the concentration range of 0.3-20 mM (Neumann *et al*.; 2006).

The recombinant AMPK heterotrimer has been shown to be activated by β-NAD$^+$ and inhibited by β-NADH *in vitro* (Rafaeloff-Phail *et al.;* 2004). This effect depends on the concentrations of AMP and ATP; increasing the concentration of AMP increased the potency of NADH to inhibit

AMPK and slightly increased that of NAD$^+$ to activate AMPK (Rafaeloff-Phail *et al.;* 2004). The ability of NADH to inhibit AMPK is decreased with increasing concentration of ATP (Rafaeloff-Phail *et al.;* 2004). The physiological significance of this effect is unclear. The effect of NAD$^+$/NADH on covalent modification of AMPK is unknown. Neuman and co-workers observed that AMPK activity is altered by

neither NADH nor NAD$^+$ at concentrations of up to 300 mM. Therefore the originally attractive hypothesis that AMPK could act as a sensor for the cellular redox potential should be carefully re-evaluated.

There appears to be a complex relationship between AMPK activity and the cellular glycogen content. High cellular glycogen was shown to be associated with decreased basal and AICAR-stimulated α2 AMPK activity in skeletal muscle (Wojtaszewski *et al.;* 2002). Moreover, the activation of AMPK in skeletal muscle induced by AICAR or by glucose withdrawal was accompanied by a decrease in glycogen synthase activity (Wojtaszewski *et al.;* 2002; Halse *et al.;* 2003). These observations suggest a reciprocal relationship between AMPK and the cellular glycogen level. The inactivation of glycogen synthase is probably due to the direct phosphorylation of glycogen synthase by AMPK, which has been shown *in vitro* (Carling and Hardie 1989). The mechanism by which glycogen inhibits AMPK is unclear. It has been shown that glycogen does not directly inhibit AMPK activity (Polekhina *et al.;* 2003). The inhibitory effect may involve glycogen-associated phosphatases or other glycogen-associated enzymes. However, this reciprocal relationship between AMPK and glycogen has not been observed under some conditions. Aschenbach *et al* demonstrated that *in vivo* AICAR administration increased glycogen in skeletal muscle despite the activation of α-2 AMPK (Aschenbach *et al.;* 2002). These authors attributed the glycogen accumulation to increased glucose uptake because no change in the activity of glycogen synthase or phosphorylase was observed (Aschenbach *et al.;* 2002). Several naturally occurring mutations in the human AMPK γ2 gene have been shown to cause

excessive glycogen accumulation in the heart. The myocardial glycogen accumulation causes ventricular pre-excitation, the condition called Wolff-Parkinson-White syndrome, which is associated with the development of cardiac hypertrophy. Transgenic mice expressing some of these mutations (N488I, R302Q, R531G) have been generated (Arad *et al.;* 2003; Davies *et al.;* 2006; Sidhu *et al.;* 2005). All of these mice showed glycogen accumulation in the heart and cardiac hypertrophy (Arad *et al.;* 2003; Davies *et al.;* 2006; Sidhu *et al.;* 2005). The AMPK activity is decreased in the heart of R302Q-expressing mice (Sidhu *et al.;* 2005), consistent with the reciprocal relationship between AMPK and glycogen. The cardiac AMPK activity of the mice expressing the R531G mutation was found to be no different at 1 week of age and there was also no glycogen accumulation at this age (Davies *et al.;* 2006). However, glycogen accumulation was seen at four weeks of age and this was associated with decreased AMPK activity (Davies *et al.;* 2005). In contrast to these mutations, the N488I mutation is associated with the accumulation of glycogen despite the increased AMPK activity (Arad *et al.;* 2003). Thus, the precise relationship between AMPK and glycogen is unclear and requires further research.
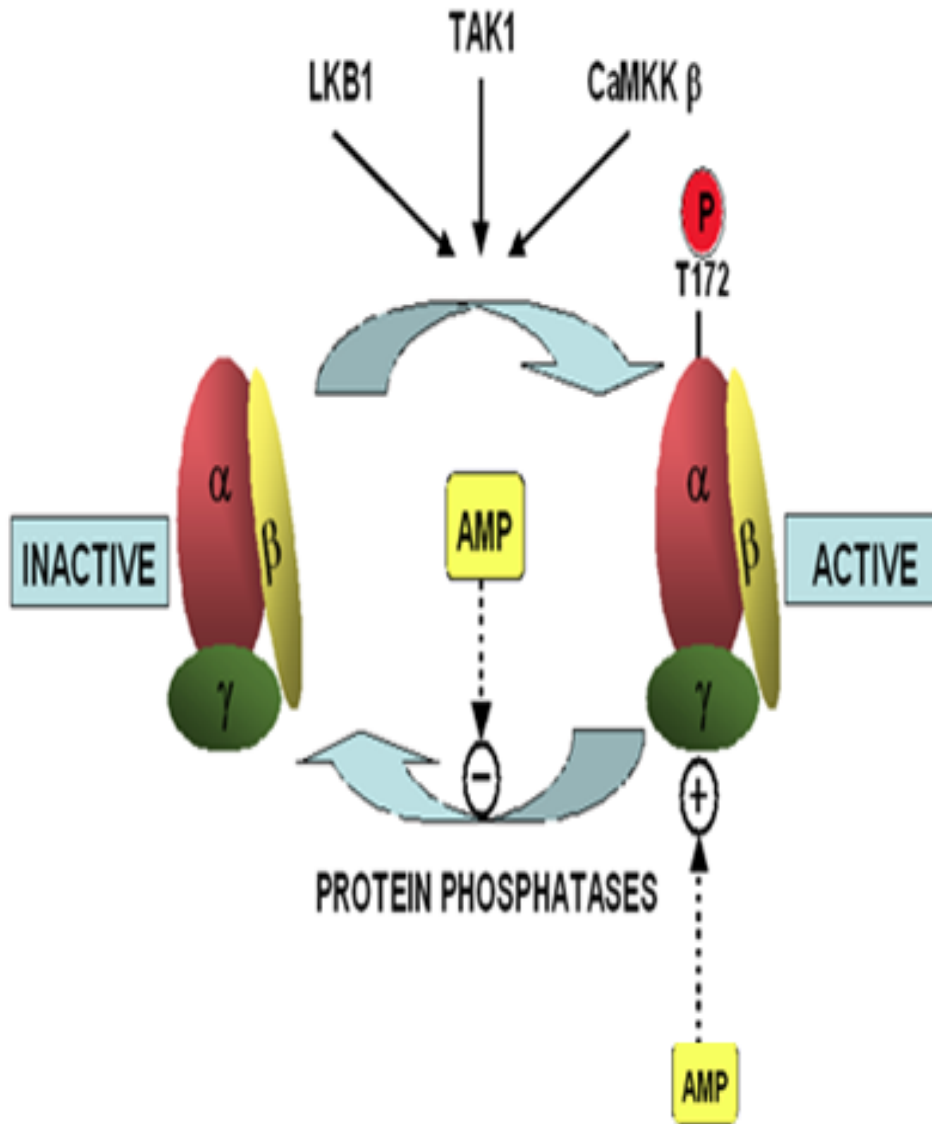
Figure 1.3: Current ideas on the regulation of AMPK activity and phosphorylation status by AMP.(Based on these publication Hardie 2007, Suter *et al*.; Sander et al.; 2007)

1.3.4 The major roles of AMPK in myocardial energy utilization

Myocardial Glucose Utilization: AMPK plays a key role in regulating cardiac glucose utilization at several levels, and stimulates glucose uptake, glycolysis (Marsin *et al.;* 2000) and possibly glycogen metabolism (Carling and Hardie 1989). AMPK promotes the translocation of GLUT4 to the sarcolemma of the myocyte in an insulin-independent manner (Bertrand *et al.;* 2006). However, it is also possible that AMPK activation decreases GLUT4 recycling from the sarcolemma, thus increasing the overall GLUT4 levels in the plasma membrane (Yang and Holman 2006). The mechanism by which AMPK activation promotes increased GLUT4 levels in the plasma membrane is not clearly defined, there is some evidence that this may be mediated by nitric oxide (Young. *et al.;* 2004) since pharmacological inhibition of eNOS and the downstream cGMP pathway partially inhibits AICAR-induced GLUT4 translocation to the plasma membrane (Young *et al*.; 2004). Besides, another study showed that NO donors inhibited AMPK activity and GLUT4 translocation to the plasma membrane in canine ischemic myocardium (Lei *et al*.; 2005). Whereas the involvement of NO in the regulation of AMPK-mediated GLUT4 translocation to the plasma membrane is controversial, it is likely that further pathways are involved. The effects of insulin and muscle contraction/cellular stress on glucose transport are additive, providing evidence for an independent pathway in the regulation of glucose metabolism (Holman *et al*.; 2006). However, both signals eventually lead to the mobilization of GLUT4 to the cell surface, which suggests that a point of convergence exists within these distinct signalling pathways. The point of the converging signalling event(s) remained unknown until it was recently realized that the *Akt* substrate AS160, a Rab-GTPase activating protein and a key regulator of GLUT4 traffic lies at a critical node and is regulated by both *Akt* and AMPK (Holman *et al*.; 2006)(**Figure1.4**).

In addition to simply providing extra substrate for glycolysis, AMPK activation promotes the production of ATP by glycolysis (Merrill *et al.;* 1997) through the direct phosphorylation and activation of heart phosphofructokinase PFK-2 at Ser466 (Marsin *et al*.; 2000). This enzyme produces fructose 2,6-bisphosphate, which is a potent stimulator of glycolysis. Together, both the direct and indirect activation of glycolysis by AMPK further increases the supply of pyruvate for glucose oxidation (Depre *et al*.; 1998). As previously mentioned, when the energy demands of the heart are fulfilled, excess glucose can be stored in the form of glycogen. This process is regulated primarily by the enzyme glycogen synthase. AMPK has been shown to inhibit glycogen synthase activity *in vitro* (Richter *et al*.; 2002) and it is expected that, during times of diminished ATP levels, there is a reduction in intracellular glycogen content *in vivo*. The effect that AMPK may have on glycogen synthase would ultimately increase the ATP supply by directing glucose to the energy producing pathways. Despite this rationale, based on *in vitro* evidence showing that AMPK activation may inhibit glycogen synthesis, the *in vivo* data do not necessarily support this function for AMPK in the heart (Longnus *et al*.; 2003). Indeed, glycogen levels are lower or unchanged in transgenic hearts possessing impaired AMPK activity compared to hearts from wild type mice (Young *et al*.; 2004).

Myocardial Fatty Acid Utilization: Fatty acids are imported into the cardiac myocyte and oxidized in the mitochondria to produce ATP. Utilization of fatty acids by heart appears to be mediated by AMPK via number of processes. For instance, the activation of AMPK has been shown to be involved in the recruitment of cardiac lipoprotein lipase (LPL) to the endocardial surface, thus promoting fatty acid availability to the heart (An D *et al*.; 2005). Furthermore, an evidence has shown that AMPK activation induced by myocyte contraction can mediate translocation of CD36 (a fatty acid transport protein) from the intracellular compartments to the plasma membrane, suggesting that AMPK is also involved in the regulation of fatty acid transport (Luiken *et al.;* 2003). In addition to increasing fatty acid levels within the cardiac myocyte, AMPK activation also increases fatty acid oxidation for the generation of ATP (Kudo *et al.;*

1995). This occurs through the phosphorylation and subsequent inhibition of acetyl CoA carboxylase (ACC) (Witters and Kemp 1992). ACC produces malonyl CoA, which is an inhibitor of carnitine palmitoyl transferase (CPT1), the enzyme responsible for the regulation of the import of long chain fatty acyl units into the mitochondria. As a consequence of AMPK activation, a lower malonyl CoA level relieves the inhibition of CPT1, accelerates fatty acyl unit transport into the mitochondria and results in the enhanced β-oxidation of fatty acids and subsequent ATP generation (Hall J *et al.;* 1996). Therefore, by promoting fatty acid availability, fatty acid transport and fatty acid oxidation, AMPK is a central mediator of fatty acid utilization in the heart (Saggerson and Clark 2004).

Glucose and fatty acid are the major fuels for the heart. Glucose has been shown to decrease α-Thr172 phosphorylation and AMPK activity in pancreatic ß-cells and skeletal muscle (da Silva Xavier *et al* 2000 and 2003; Itani *et al*.;2003; Halse *et al*.; 2003). By contrast, physiological concentrations of long chain fatty acids (LCFA) such as palmitate have been shown to increase α-Thr172 phosphorylation and to activate AMPK in heart and skeletal muscle (Hickson-Bick *et al* 2000; Clark *et al* 2004; Fediuc *et al* 2006; Watt *et al* 2006), although this effect has not been observed by some investigators (Olsen and Hansen 2002; Dobrzyn *et al*.; 2005; Crozier *et al*.; 2005; Folmes *et al*.; 2006). The following sections will focus on the effect of glucose, fatty acids and hormones on AMPK during physiological and pathological conditions.
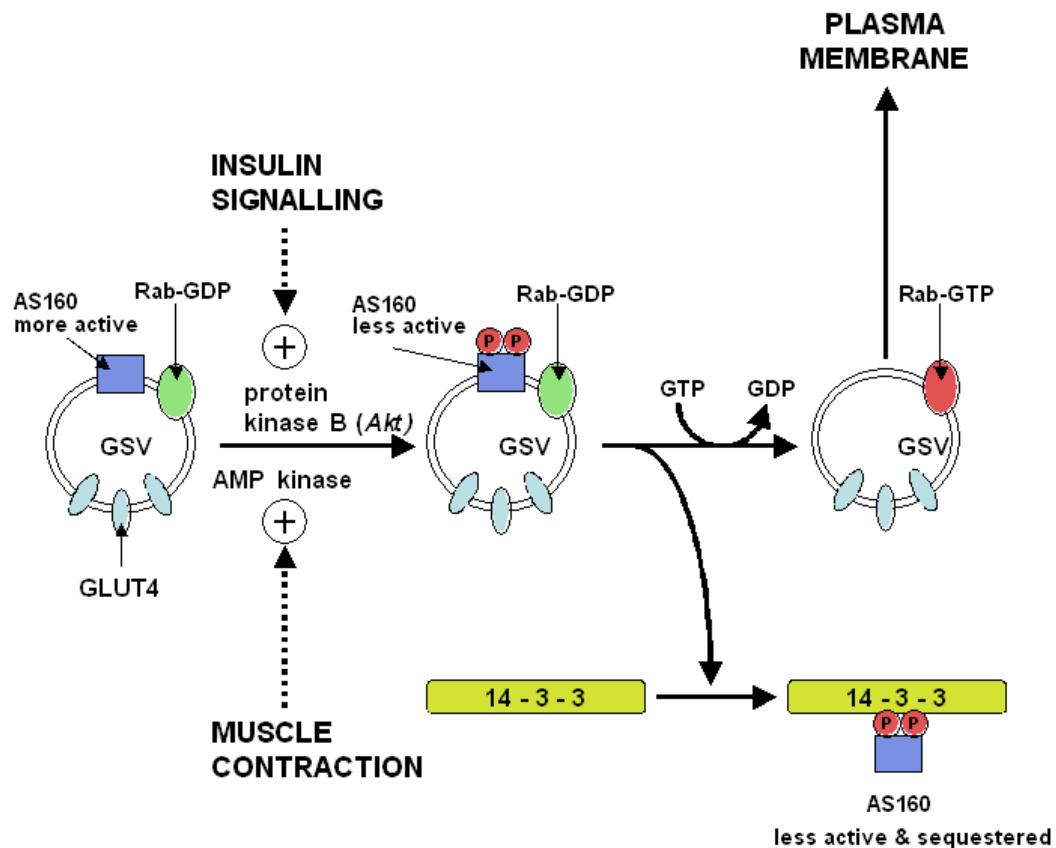
Figure 1.4 Insulin leads to autophosphorylation of its receptor which, in turn, leads to tyrosine phosphorylation of IRS. This couples to the activation of PI 3-kinase (phosphoinositide 3-kinase) which produces PIP3 (phosphatidylinositol trisphosphate) in the plasma membrane. PDK1, PDK2 and *Akt* become associated with PIP3 and together lead to the phosphorylation and activation of *Ak*t. Activated *Akt* then phosphorylates ASI60 and this leads to the suppression of its GTPase-activating activity. This may occur directly or indirectly through the association of the phosphorylated proteins (Rab-GAP-p) with 14-3-3 protein and relocation to the cytoplasm. Following the reduction in GLUT4-vesicle-associated Rab GAP activity, the vesicle-associated Rabs are converted into their active GTP-loaded forms and this switch facilitates the trafficking of the vesicles to the plasma membrane and possibly enhanced docking at the plasma membrane. AS160 is a key regulator of GLUT4 trafic which is regulated by both AMPK and *Akt.*(Adapled from Koumanov& Holman 2007 , sakamato & Holman 2008)

1.4 The glucose fatty acid cycle (Randle Cycle)

The Randle cycle has been invoked to explain the reciprocal relationship between fatty acid oxidation and glucose oxidation which, simply stated, says that increased fatty acid oxidation causes a decrease in glucose oxidation leading to a decrease in glucose uptake (Randle *et al*., 1963). The oversupply of lipid to the muscle increases the amount of lipid-derived acetyl-CoA. A high [acetyl- CoA]/ CoA] ratio in mitochondria inhibits the pyruvate dehydrogenase complex, and increases citrate concentration. An elevated cytoplasmic citrate level inhibits phosphofructose-kinase-1 activity and causes the accumulation of glucose-6-phosphate, which eventually leads to a feedback inhibition of glucose uptake **Figure 1.5**. Shulman's group using the nuclear resonance technique NMR, showed that, after lipid-heparin infusion to increase plasma NEFA concentration, glucose oxidation and muscle glycogen synthesis were 50-60% lower than in the control group, which is consistent with Randle's theory. Oon the contrary Shulman suggests that the initial inhibition occurs at the glucose transport step instead of pyruvate dehydrogenase. This is supported by the observation of decreased intracellular glucose and glucose-6-phosphate levels during lipid infusion (Dresner *et al*.; 1999). However, whether the defect in glucose transport is a direct effect of NEFA on glucose transporter trafficking or an indirect effect through upstream signaling or both remains unclear (Pfanner *et al.;* 1989, 1990). The NEFA and their metabolites also directly interfere with the insulin signaling pathways. Lipid infusion into a of muscle biopsy resulted in a decrease in IRS-1 associated PI-3 kinase activity in parallel to diminished IRS-1 tyrosine phosphorylation, possibly due to the activation of PKC and other Ser/Thr kinase pathways by increasing intracellular fatty acyl-CoA and its metabolites, such as diacylglycerol (Dresner *et al*.; 1999). This leads to Ser/Thr phosphorylation of the insulin receptor and IRS-1 (Nishizuka *et al*.; 1995; Schmitz-Peiffer.; 2002). The PKC-mediated serine phosphorylation of IRS-1 inhibits its tyrosine phosphorylation by the insulin receptor kinase and its downstream signaling to PI-3 kinase (Griffin, *et al*.; 1999). Fatty acid metabolites can activate pathways that lead to the down regulation of insulin stimulated glucose disposal in muscle. The

NEFA, mainly, oleate, palmitate and linoleate, are converted to long chain fatty acyl-CoA. Palmitate only serves as a substrate for the *de novo* synthesis of ceramide, which appears to interfere with insulin signaling through decreasing Akt/PKB phosphorylation (Chavez *et al*.; 2003). As the major insulin-stimulated site of glucose uptake, skeletal muscle is of particular importance. Experiments with lean normoglycemia offsprings of parents with Type 2 diabetes mellitus (T2DM) showed insulin resistance in muscle before the onset of frank hyperglycemia, indicating alteration of glucose metabolism in muscle, is an early event in the pathogenesis of T2DM (Rothman *et al*.; 1995). However, this has long been implicated as a potential mechanism for hyperglycemia and T2DM.

Glucose fatty acid cycle

(A)

FFA Interaction with Glucose Uptake

(B)
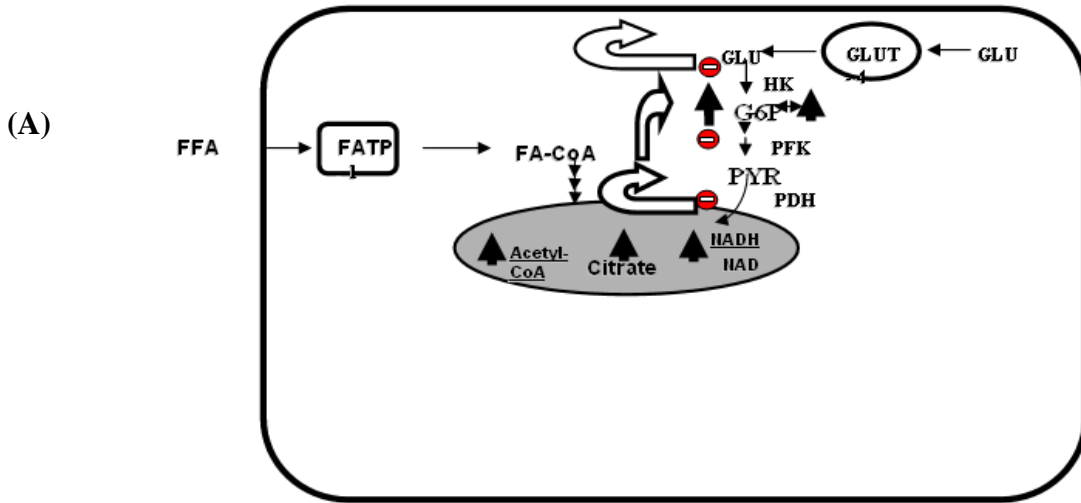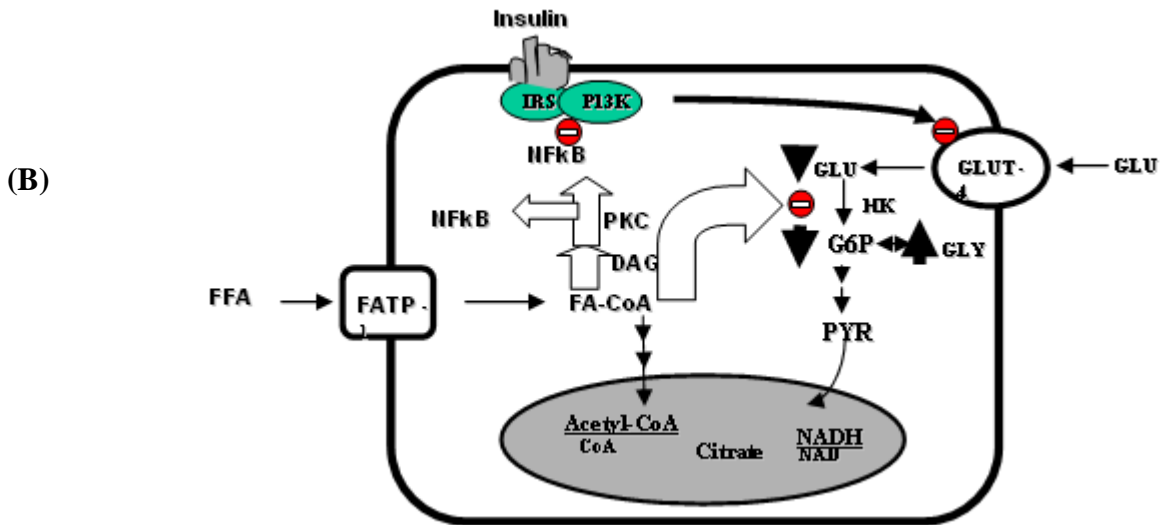
Figure 1.5 Interaction of free fatty acids (FFA) with glucose (GLU). FFA are taken up by the fatty acid transporter protein-1 (FATP-1) and activated to fatty acyl-coenzyme A (FA-CoA), whereas GLU is taken up by glucose transporter-4 (GLUT-4) and phosphorylated by hexokinase-II (HK). *A*: glucose-fatty acid cycle (Randle hypothesis). FA-CoA oxidation would increase the mitochondrial ratios of acetyl-CoA/CoA and of NADH/NAD$^+$, which inhibit the pyruvate dehydrogenase (PDH) complex. Increased citrate should further inhibit phosphofructokinase (PFK). These changes would slow down the oxidation of GLU and pyruvate (PYR) and increase glucose-6-phosphate (G6P), which would probably stimulate glycogen (GLY) storage, inhibit hexokinase (HK), and decrease GLU transport. *B*: FFA interaction with glucose uptake activates isoforms of protein kinase C (PKC) and other Ser/Thr kinase pathways.

1.5 Glucose

The rate of glucose utilization by the heart is primarily linked to the rate of glucose uptake into the cell. This process is mediated by facilitated diffusion through specific transporters, and the rate is controlled by the concentration gradient, the number of transporters and their affinity for substrate (King and Opie, 1998). Once inside the cells, glucose is phosphorylated to glucose 6-phosphate. Various metabolic factors play a role in determining the rate of glucose utilization. Such factors include dietary state, the availability of alternative substrate and the levels of secreted hormones. Glucose transport is rate limiting for glucose metabolism in unstimulated perfused rat heart (Morgan *et al*.; 1961). Increasing extracellular glucose results in an increase in glucose 6-phosphate without an increase in intracellular free glucose. Glucose fulfils multiple functions in cells that extend far beyond its use as an energy source. Metabolites driven from glucose has been found to act as signalling molecules, such as malonyl-CoA and fructose 2,6-bisphosphate. Other metabolites have an effect on gene expression. The regulation of gene expression via intracellular glucose concentrations has been observed for a number of genes products. These include glucose transport, glycolytic and lipogenic enzymes, such as pyruvate kinase and acetyl CoA carboxylase (Vaulont *et al*.; 2000). In adipose tissue and muscle, the hexosamine pathway may be responsible for some of the transcriptional effects of glucose (Sayeski *et al*.; 1996). Protein phosphatase inhibitors (eg okadaic acid) inhibit the glucose induction of liver-PK expression (Datta *et al*.; 1999). Recently, it has been demonstrated that the activation of AMPK by 5-amino-4imidazolecarboxamide riboside (AICAR) inhibits the activation of many glucose-regulated genes in primary hepatocytes (Leclerc *et al*.; 1998).

1.5.1 Glucose transport

There are two mechanisms for facilited 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. Unlike active transporters, which require energy to drive their translocation mechanism, this facilitated transport of glucose occurs down a concentration gradient.

The major glucose transporter expressed in cardiomyocytes is the GLUT4 isoform. The GLUT1 transporter, which is present in most tissues, is also found in cardic myocytes, but is only a fifth as abundant as GLUT4 (Kraegen *et al.;* 1993). GLUT1 has higher affinity for glucose and is likely to be accountable for efficient transport at low circulating glucose concentrations. At normal cardiac workload, circulating insulin regulates the rate of glucose extraction by cardiomyocytes (Taegtmeyer *et al.*; 1985). An increase in workload increases glucose uptake in an insulin independent manner, by a mechanism which is mediated by AMPK-activated protein kinase. In the absence of insulin, glucose transport is the rate limiting step in the utilization of exogenous glucose in normoxic perfused hearts (Kobayashi & Neely, 1979). In the presence of insulin, glucose transport is no longer rate-limiting and imposes little restraint on glucose uptake and utilization. (Kobayashi & Neely 1979).

A number of other factors are also known to increase the rate of glucose transport in heart muscle, including hormones, such as insulin, a lack of oxygen or increasing the energy demand of the heart (during exercise), and it is decreased by alternative substrates, such as fatty acid and ketone bodies. The products of intermediary metabolism have been shown to decrease glucose transport in isolated cardiac myocytes, such as pyruvate, lactate and propionate (Fischer *et al.*; 1998).

1.5.2 Glycolysis

Glycolysis is a metabolic pathway that is found in the cytosol of cells in all living organisms. The process converts one mol of glucose into two mol of pyruvate, with a net yield of two mol of ATP. Actually four molecules of ATP per glucose are produced; however, two are consumed

during the preparatory phase. The initial phosphorylation of glucose is required to destabilize the molecule for cleavage into two triose sugars. During the pay-off phase of glycolysis, four phosphoryl groups are transferred to ADP by substrate-level phosphorylation to make four ATP, and two NADH are produced when the triose phosphate are oxidized. The overall reaction can be expressed as follows:

Glucose + 2 NAD$^+$ + 2 P$_i$ + 2 ADP $\rightarrow$ 2 pyruvate + 2 NADH + 2 ATP +2H$^+$ + 2 H$_2$O

The principal function of glycolysis is to produce ATP. Therefore it must be regulated so that ATP is generated only when needed. In the normally oxygenated heart, the amount of glycolytically-generated ATP is small compared to that produced by oxidative phosphorylation in the mitochondria. Aerobic glycolysis has been shown to be critical for normal cardiac excitation-contraction (E–C) coupling. The inhibition of aerobic glycolysis may lead to the decline of excitability and [Ca^{2+}]$_i$ transients in cardiac myocytes. The strict functional coupling between glycolysis and E–C coupling is thought to be due to a close association of glycolytic enzymes and ion channels, transporters, and pumps participating in the E–C coupling process (Han *et al;.* 1992; Xu *et al.;* 1995). A number of membrane transport systems are regulated preferentially by glycolytically-derived ATP, including the sarcolemmal K$_{ATP}$ channel (Weiss & Lamp, 1987, 1989), voltage-gated Ca^{2+} channels (Losito *et al.;* 1998), the Na$^+$–H$^+$ exchanger (Wu & Vaughan-Jones, 1994), the Na$^+$–K$^+$ pump (Glitsch & Tappe, 1995), and the sarcoplasmic reticulum (SR) Ca^{2+} pump (Xu *et al.;* 1995).

In addition, some studies also revealed that the ion channels involved in E–C coupling may be modulated directly by the intermediates of the glycolytic pathway. For example, the ryanodine receptor (RyR), the Ca^{2+} release channel of the SR, was found to be stimulated by glucose and fructose phosphates (Kermode *et al.;* 1998). Also it was suggested that the activity of the sarcolemmal Na$^+$ channel is modulated directly by 2,3-bisphosphoglycerate and glyceraldehyde 3-

phosphate (Kohlhardt *et al.;* 1989), while later studies found no evidence for the direct modulation of the Na$^+$ channel when the myocardial fuel metabolism is tightly regulated and closely parallels the rate of cardiac work. However, under pathological conditions, the significance of glucose metabolism increases. Hypertrophy is known to increase the reliance of the heart on glycolytic metabolism. Conversely, diabetes results in a major decline in cardiac glycolysis (Luigino *et al*.; 2004) that is associated with diabetic cardiomyopathy. In ischemic hearts, the AMPK signaling pathway stimulates glucose transportation and PFK-2 by phosphorylating it at Ser466 (Lopaschuk *et al.;* 2008). This stimulates glycolysis. In fact, the stimulation of glycolysis with glucose and insulin has been used successfully to protect patient hearts from ischemic damage. A number of reports have suggested that ATP derived from glycolysis is preferentially utilized to support membrane functions in the heart (Terence *et al.;* 1973)

1.5.2.1 Hexokinase

Glucose phosphorylation by hexokinase is the initial regulatory step that commits glucose to further metabolism. Two different isozymes of hexokinase are present in the heart, hexokinases I and II. (Printz *et al.;* 1993) Hexokinase I is predominant in the foetal and newborn heart. The phosphorylation of glucose by hexokinase maintains intracellular free glucose concentrations at a low level, providing the concentration gradient required for sustained glucose flux into the myocytes. Hexokinase is therefore necessary for the regulation and continuance of myocardial glucose uptake (Wilson *et al.;* 2003). Insulin-responsive tissues express two hexokinase isoforms, hexokinase I (HK I) and hexokinase II (HK II) (Burcelin *et al.;* 1993). Under physiological conditions, HK II is mainly located in the cytosol (Foucher *et al.*; 1984), whereas HK I is mainly bound to the mitochondria (Lynch *et al.;* 1996, Ritov *et al.;* 2001). The binding of hexokinase to the mitochondria is thought to make hexokinase less susceptible to product inhibition (Hue *et al*.; 1998), providing hexokinase with better access to mitochondrially produced ATP (Depre *et al.;*

1998, Wilson *et al.*; 1997), and providing the mitochondria with ADP for oxidative phosphorylation (Penso *et al.*; 1998, Sothworth *et al.;* 2007). The interface of hexokinase with the mitochondria has also been proposed to play beneficial nonenzymatic roles in cellular protection by decreasing mitochondrial superoxide leakage (Da Silva *et al.;* 2004) and preventing apoptosis (Zohar , *et al.;* 2004, Hay *et al.;* 2004).

In skeletal muscle, insulin has been shown to promote the translocation of HK II from the cytosol to mitochondrial membranes (Vogt *et al.;* 2000). Thus, insulin's effect of increasing glucose uptake by externalizing GLUT4 is complemented by an increased capacity to phosphorylate glucose by insulin-mediated hexokinase translocation to the mitochondria. This double response means that the limit at which hexokinase becomes a rate-limiting step to glucose accumulation is raised (Fueger *et al.*; 2003). Whether cardiac hexokinase also responds to insulin in this manner is presently controversial, with the most recent study concluding that cardiac hexokinase translocation does not occur (Southworth**,** *et al.;* 2007, Vogt *et al.;* 1998). Although GLUT4 is known to translocate to the cardiac sacrolemma during ischemia, no studies to date have investigated whether hexokinase also translocates to the mitochondria to sustain cardiac glucose accumulation.

Characterizing hexokinase behaviour in tissue is problematic because its activity is directly linked to its intracellular location and chemical microenvironment. Assessing hexokinase activity in tissue homogenates, as has been done in the past (Lawrence and Trayer 1985, Ritov and Kellek 2001), is likely to have limited relevance to hexokinase activity *in situ*, since hexokinase-mitochondrial interaction is likely to be disrupted or absent. An estimation of the extent of mitochondrial binding by cellular fractionation has also been shown to be of limited value. Although the extent of hexokinase-mitochondrial binding in astrocytes by fractionation has been measured as 15–40%, parallel studies in an intact cell population by confocal microscopy showed that ∼70% of hexokinase was mitochondrial bound (Lynch *et al.;* 1991). Tissue homogenization

would thus appear to lead to a dissociation of hexokinase from the mitochondria and invalidate the results. Heat lability, used in the past to discriminate between HK I and HK II isoforms, has also been discredited. Because hexokinase lability is highly influenced by a number of factors,

including solvent conditions, the presence of other proteins, and in particular mitochondrial binding, the use of this technique to distinguish the isoforms is imperfect (Ritov *et al*.; 2001, Wilson *et al*.; 2003).

1.5.2.2 Phosphofructokinase –1

Phosphofructokinase-1 (PFK-1) is a key enzyme in glucose metabolism. It catalyses the second irreversible step of glycolysis to generate fructose 1,6-bisphosphate. PFK-1 is a tetrameric enzyme composed of four identical subunits. Like other allosteric proteins (haemoglobin) and enzymes such as aspartate transcarbamoylase (ATCase), the binding of allosteric effectors and substrates is communicated to each of the active sites. Quaternary changes are concerted and preserve the symmetry of the tetramer. PFK-1 has two sets of alternative interactions between the subunits which are stabilized by hydrogen bonds and electrostatic interactions. The two set of conformations are called the T and R states. These two conformational states are in equilibrium: T – R

ATP is both a substrate and an allosteric inactivator of PFK-1, which has two binding sites for ATP. One is the substrate binding site and the other is an allosteric inhibitory site. The PFK-1 substrate binding site binds ATP equally well in both the T and R states. The inhibitory ATP binding site only binds ATP when the enzyme is in the T formation. The other substrate fructose-6-phosphate binds only to the R state. High concentrations of ATP shift the equilibrium towards the T conformation which decreases the affinity of the enzyme for F6P. AMP binds preferentially to the R state of PFK-1. AMP concentration levels in the cell can rise due to the enzymatic activity of adenylate kinase. The steady state concentration of ATP in the cell is normally 4 times

greater than the concentration of ADP, and normally 20 times the concentration of AMP. As a result of the activity of adenylate kinase, a 10% decrease in the concentration of ATP can result in a 400 % increase in the concentration of AMP.

Other allosteric effectors of PFK-1

ADP is another allosteric effector of PFK-1. ADP, like AMP, reverses the inhibitory effects of ATP and is therefore considered an allosteric activator. The activity of PFK-1 is dependent on the ATP, ADP and AMP concentrations which play an essential role in the cellular energy status. Under aerobic conditions, the pyruvate formed by glycolysis is fed into the citric acid cycle where it is completely oxidized to CO_2 and H_2O. Citrate is a metabolite of the citric acid cycle. When acetyl-CoA provision exceeds the need of the citric acid cycle, the citrate concentration in mitochondria and the cytosol increases, which leads to a decrease in glycolytic flux. Citrate binds preferentially to the T state of PFK-1. Thus, high concentrations of citrate inactivate the enzyme. PFK-1 is also potently activated by fructose 2-6-bisphosphate. Fructose 2-6-bisphosphate binds to the R-state of the enzyme and increases the affinity of the enzyme for fructose-6-phosphate.

The accumulation of AMP decreases the sensitivity of PFK-1 to $[H^+]$. PFK-1 activity is sensitive to intracellular pH, especially over the physiological range pH 6.8 to 7.3. Studies using purified heart enzyme reveal a sigmoidal relationship between enzyme activity and the concentration of F6P at pH 6.9, and a marked sensitivity to allosteric regulation. At pH 8.2, PFK-1 exhibits Michaelis-Menten kinetics, has increased activity, and is not subject to allosteric regulation (Trivedi & Danforth, 1966). The product of the PFK-1 reaction, fructose 1,6-bisphosphate (F-1,6BP), a potent inhibitor of the enzyme PFK-1, has two binding sites , a low affinity site and a high affinity site (Proffit *et al*.; 1976). Binding to the low affinity site probably accounts for allosteric regulation at pH 6.9, while binding to the high affinity site affects catalytic activity even at pH 8.2. Adrenaline increases PFK-1 activity in muscle (Tag *et al*.; 1972), which may be the

result of the more extensive binding of the effectors, especially F1 ,6BP. Adrenaline also stimulates PFK-1 activity in rat heart by Ca^{2+} - dependent α- and β-adrenergic mechanisms (Clark & Patten 1984).

In vitro studies of cardiac PFK-1 have shown it to be a substrate for cyclic AMP-dependent (PKA) and Ca^{2+}/calmodulin-dependent (CAMK) protein kinases (Mahrenholz *et al*.; 1991). Phosphorylation resulted in an increase in the sensitivity of PFK-1 to inhibition by ATP as well as an increase in its K_m for F6P. These effects are minor, compared to the allosteric effects and it is unknown whether the phosphorylation of cardiac PFK-1 has a regulatory role *in vivo.*

1.5.2.3 Fructose 2, 6-bisphosphate

Fructose 2,6-bisphosphate (F2,6BP) most convincing potent activator of phosphofructokinase-1. The synthesis and degradation of this unique sugar bisphosphate are controlled by the bifunctional enzyme fructose 6-phosphate 2-kinase and fructose 2,6-bisphosphatase. Various tissues contain tissue-specific isozymes of the bifunctional enzyme, and these isozymes have different relative kinase and phosphatase activites (Uyeda *et al*.; 1991). Several of these isozymes from various mammalian tissues have been characterized. They are all homodimers, consisting of subunits with Mr ranging from 54,000 to 60,000 Da. The amino acid sequences of the enzymes from skeletal muscle (Crepin *et al*.; 1992), the heart (Sakata & Uyeda, 1990), the liver (Lively *et al*.; 1988), the testis (Sakata *et al.;* 1991), and the brain (Watanabe *et al*.; 1994) have been determined. The amino acid sequences of both catalytic domains are highly conserved among these isozymes, but the amino and carboxyl termini are completely different. These differences in the terminal peptides appear to regulate the relative activities of the kinase and the phosphatase, which in turn regulate the F2,6BP concentrations in each tissue. The activity ratio of the enzyme can also be modulated by phosphorylation/dephosphorylation. The phosphorylation of the C-terminus of the heart enzyme results in the activation of the kinase without any effect on phosphatase activity (Kitamura & Uyeda, 1987; Kitamura *et al*.; 1988). In contrast, the

phosphorylation of the N-terminus of the liver enzyme results in the inhibition of the kinase and the activation of the phosphatase (Van Shaftingen *et al*.; 1981; Maghrabi *et al*.; 2001).

 Some isozymes including the rat testis bifunctional enzyme lack such phosphorylation sites. It is known that the kinase domain resides in the amino-terminal half and the phosphatase domain in the carboxyl-terminal half of the enzyme. Heart PFK-2 activity is inhibited by citrate and *sn*-glycerol-3-phosphate (Hue & Rider, 1987) and activated by insulin. Insulin activates PFK-2 possibly through the phosphatidylinositol 3-kinase pathway (Hue *et al*.; 1995). Conditions which reduce glycolysis, such as the oxidation of fatty acids, ketone bodies or lactate, are citrate mediated and accompanied by decreases in F 2,6BP2, levels (Hue *et al*.; 1995), whereas the stimulation of glycolysis by glucose, insulin, adrenaline or increased workload is paralleled by an increase in the F $2,6BP_2$ content of isolated perfused hearts (Hue & Rider 1987). F 2,6BP2 can therefore act as an interacellular signal to control glycolysis in various tissues under normoxic conditions (Hue & Rider, 1987).

Heart PFK-2 was phosphorylated on Ser466 and activated by AMPK *in vitro*. In perfused rat hearts, anaerobic conditions or the inhibitors of oxidative phosphorylation (oligomycin and antimycin) induced AMPK activation, which correlated with PFK-2 activation and with an increase in fructose 2,6-bisphosphate concentration. Moreover, in cultured cells transfected with heart PFK-2, oligomycin treatment resulted in a parallel activation of endogenous AMPK and PFK-2 (Hue *et al.;* 2000). In these cells, the activation of PFK-2 was due to the phosphorylation of Ser466. A dominant-negative construct of AMPK abolished the activation of endogenous and cotransfected AMPK, and prevented both the activation and phosphorylation of transfected PFK-2 by oligomycin. AMPK-mediated PFK-2 activation is likely to be involved in the stimulation of heart glycolysis during ischaemia (Hue *et al*.; 2000).

1.5.2.4 Glyceraldehyde-3-phosphate Dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an enzyme involved in glycolysis, which catalyzes the NAD^+-dependent oxidation of glycerol 3-phosphate (G3P) to 1,3-bisphosphoglycerate (1,3-BPG) and NADH. This is the first step in which a high energy phosphate compound (acyl phosphate) is generated. GAPDH is inhibited by its product, 1,3-bisphosphoglycerate and NADH, and by lactate (Mochizuki & Neely, 1979) and ATP (Meriwether & Park, 1971). Activation of PFK-1 leads to an accumulation of Fru 1,6-P2 and, under specific situations, to a restriction of glycolytic rate further down the pathway. The control of glycolytic rate is shifted from PFK-1 to GAPDH in hearts perfused under anoxic or ischaemic conditions, resulting in the accumulation of F1,6BP2 and triose phosphate (Eaton *et al*.; 2002) Under ischemic or anoxic conditions, the reoxidation of NADH to NAD^+ is impaired (Eaton *et al.;* 2002). The resulting increase in NADH inhibits GAPDH. 1, 3-bisphosphoglycerate is used to generate the first molecule of ATP from glycolysis by the transfer of the phosphoryl group from the acyl phosphate of 1, 3- bisphosphoglycerate to ADP, a reaction catalysed by phosphoglycerate kinase.

Recent evidence suggests that mammalian GAPDH is also involved in a great number of intracellular processes, such as membrane fusion, microtubule bundling, phosphotransferase activity, nuclear RNA export, DNA replication and DNA repair (Hessler *et al.;* 1998). During the last decade, many findings have been made concerning the role of GAPDH in different pathologies, including prostate cancer progression, programmed neuronal cell death and age-related neuronal diseases, such as Alzheimer's and Huntington's. GAPDH is constitutively expressed in almost all tissues at high levels. There are, however, some physiological factors, such as hypoxia and diabetes that increase GAPDH expression in certain cell types.

1.5.2.5 Pyruvate Kinase

Pyruvate kinase is the enzyme catalyses the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP, yielding one molecule of ATP and one molecule of pyruvate.The heart isoform is inhibited by ATP. This is similar to the effect of ATP on PFK-1. The binding of ATP to the inhibitor site reduces its affinity for PEP. The liver enzyme is also controlled at the level of synthesis. Increased carbohydrate ingestion induces the synthesis of PK, resulting in elevated cellular levels of the enzyme. This slower form of control, which responds to long-term dietary and environmental factors, is referred to as transcriptional regulation.

The majority of pyruvate produced from glycolysis in heart and skeletal muscle is transported into the mitochondria, where it can be converted to acetyl-CoA by the action of pyruvate dehydrogenase (PDH). When oxygen is lacking, NADH accumulation causes inhibition of PDH and the citric acid cycle and pyruvate is converted to lactate by lactate dehydrogenase. The conversion of pyruvate to lactate as opposed to acetyl-CoA depends upon the oxidation state of the cell, the level of NADH and acetyl-CoA and PDH activity.

1.5.2.6 Pyruvate dehydrogenase

PDH complex is one of the main control points in carbohydrate metabolism. It exists in both active and inactive forms, which are interconvertible by phosphorylation and dephosphorylation. It is located in the mitochondrial matrix of eukaryotes. The pyruvate dehydrogenase complex catalyzes the oxidation and decarboxylation of pyruvate to form acetyl-CoA, NADH and CO_2. The mammalian PDH complex contains multiple copies of pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2) and dihydrolipoamide dehydrogenase (E3), which together catalyse the decarboxylation reactions, two regulatory enzymes (PDH kinase and PDH phosphatase) and protein X which links E3 to the complex.

Pyruvate dehydrogenase (E1)

Pyruvate dehydrogenase (E1) is the first component enzyme of the pyruvate dehydrogenase complex (PDC) and performs the first two reactions within the PDC: a thiamine pyrophosphate-dependent decarboxylation of substrate 1 (pyruvate) and a reductive acetylation of substrate 2 (lipoic acid). Lipoic acid is covalently bound to dihydrolipoamide acetyltransferase (E2), which is the second catalytic component enzyme of PDC. The phosphorylation of E1 by pyruvate dehydrogenase kinases inactivates E1 and subsequently the entire complex. This is reversed by pyruvate dehydrogenase phosphatase. Pyruvate dehydrogenase kinase activity is stimulated by acetyl-CoA and NADH (Kerbey *et al.;* 1976) and inhibited by pyruvate and the pyruvate.

Dihydrolipoyl transacetylase (E2)

Interaction of the three enzyme are affected by lipoate bound covalently to the mobile arm of E2 which visitis the active site of the three enzymes sequentially (Reed , 1969) and transfers acetyl (to CoASH) and $2e^-$ via the flavoprotein E3 to NAD^+

. Dihydrolipoyl dehydrogenase (E3)

The lysine residue of the comples bound to the dihydrolipoate then migrate to the dihydrolipoyl dehydrogenase (E3) active site, where it undergoes a flavin-mediated oxidation, identical in chemistry to disulfide isomerase. First, FAD oxidizes the dihydrolipoate back to its lipoate resting state, producing $FADH_2$. Then, NAD^+ oxidizes $FADH_2$ back to its FAD resting state, producing NADH.

Regulation

PDH kinase activity is stimulated by increasing the mitochondrial ratios of acetyl-CoA/CoASH, $NADH/NAD^+$ and ATP/ADP (Kerbey *et al.;* 1976). The activating effects of acetyl-CoA and

NADH involve the reduction of the lipoyl moiety of the complex, and are antagonised by CoASH and NAD^+. PDH kinase is inhibited by pyruvate and the pyruvate analogue dichloroacetate (McVeigh and Lopaschuk 1990). The stimulatory effect of pyruvate on PDH activity can be overcome by fatty acids and is impaired in diabetes. The oxidation of fatty acids and ketone bodies decreases the amount of PDH in its active form. Fatty acid oxidation results in increases in the mitochondrial $NADH/NAD^+$ and acetyl-CoA/CoA ratios, which are able to cause the feedback allosteric inhibition of PDH, as well as resulting in the activation of PDH kinases and conversion of PDH to the inactive phosphorylated form. PDH phosphatases are dependent upon Mg^{2+} and is activated by a physiological concentration of Ca^{2+} (0.1-10 µM). The perfusion of isolated hearts with increasing Ca^{2+} concentrations has been shown to increase the amount of PDH in the active dephosphorylated form (Pettit *et al.;* 1972). The perfusion of rat hearts with adrenaline increases the activity of PDH, probably by increasing the cellular Ca^{2+} levels, which, in turn, activates PDH phosphatase. In adipose tissue, PDH is activated by insulin, whilst in the heart no effects of insulin have been observed.

Increased cardiac work may also decrease the mitochondrial acetylCoA/CoASH and $NADH/NAD^+$ ratio, thereby relieving the inhibition of PDH by its phosphorylation by PDH kinases. In addition, during starvation, PDH kinase activity and expression (PDH kinase 4) increases in amount in most tissues, including skeletal muscle. These changes together with a shift in metabolism toward fatty acid oxidation the fasting state greatly decrease the muscle to catabolize glucose whilst increasing the output of the gluconeogenic precursor alanine. Such glucose as is available under these conditions is spread for use by the brain or by other tissue for which glucose is essential.

15.2.7 Pyruvate

Pyruvate, a product of glycolysis it has been shown to improve the mechanical performance of the heart under normoxic conditions (Zweier & Jacobus, 1987) as well as during ischaemia-

reperfusion injury (Mallet *et al.;* 1994) Current studies have suggested that pyruvate is able to potentiate the positive inotropic effects of β-adrenergic stimulation (Hermann *et al*.; 2000) and to improve the contractile performance of isolated failing human In spite of growing agreement about the beneficial effects of pyruvate on cardiac performance, the cellular mechanisms of pyruvate action have remained uncertain or even contradictory. There is, however, solid support that pyruvate improves the energetic conditions of cardiac cells. With normal oxygen supply, pyruvate undergoes oxidation in the mitochondria, leading to the augmentation of the $NADH/NAD^+$ ratio and therefore to an increase in the energetic force for ATP synthesis (Laughlin & Heineman, 1994) The uptake of pyruvate into cardiac myocytes is mediated by a sarcolemmal H^+-monocarboxylate symporter (Poole & Halestrap, 1993). Thus, pyruvate uptake is likely to cause intracellular acidification, which, in turn, could affect contractility in multiple ways (Hasenfuss *et al.;* 2002).

Aside from its beneficial effects on cardiac performance, pyruvate may also exert pro-arrhythmogenic effects by causing cardiac alterations. Recent data suggest that pyruvate can elicit alternations in the $[Ca^{2+}]_i$ transient amplitude, contraction strength, and action potential duration in both atrial and ventricular myocytes from the cat heart (Hüser *et al.* 2000; Kockskämper & Blatter, 2002). Pyruvate induced Ca^{2+} alterations was particularly noticeable in the atrial myocytes, where it caused large subcellular inhomogeneities and could produce arrhythmogenic Ca^{2+} waves.

1.5.2.8 Lactate

Lactate is found in essential cells of the body but particularly in muscle cells during exercise. Accumulation occurs when the supply of oxygen to the cells is restricted either because the muscle cells are working so hard that the oxygen supply cannot keep up or because the supply itself is lacking for some reason.

Throughout everyday activities, such as walking, the oxygen supply to muscle cells is sufficient to enable the cells to make use of energy from various sources without recourse to the lactic acid route. However, when there is an unexpected need for excessive muscular effort lactic acid formation is available to enable activity to continue, even though the oxygen supply is inadequate. This is known as the anaerobic phase of exercise in contrast to the aerobic phase which implies a sufficient oxygen supply to the muscle cells. Under normal conditions, lactic acid, an important respiratory substrate for the heart, is transported into the cardiac myocyte and oxidized. In contrast, lactic acid must be transported out of the cell to prevent intracellular acidification during hypoxia or any othercondition that increases lactic acid production or diminishes intracellular oxidation of the molecule.

1.5.3 The pentose phosphate pathway

The pentose phosphate pathway (also called the Hexose Monophosphate Shunt [HMP shunt] or Phosphogluconate Pathway) is a process that synthesizes pentose (5-carbon) sugars and serves to generate NADPH. The pathway consists of two distinct phases. NADPH is generated by the oxidative arm. 5-carbon sugars are also generated by the oxidative phase, and the non-oxidative phase is a potential alternative way of making these sugars (Heckmann and Zimmer 1992)(**Figure 1.6**) Ribulose 5- phosphate is formed through the oxidation subsequent decarboxylation of the glucose 6-phosphate. Ribulose 5-phosphate can be converted by phosphopentose isomerase to ribose 5-phosphate. The oxidation and decarboxylation of glucose-6-phosphate to ribulose-5-phosphate occurs in three steps, accompanied by the generation of two mol of NADPH. The first step is the oxidation of the hydroxymethylene group at position one to a carbonyl group, (cyclic ester) and a molecule of NADPH. The second step is then to hydrolyze the lactone to the free carboxylic acid. The carboxyl group of the carboxylic acid is then removed by oxidative

decarboxylation, converting the 6-carbon sugar acid to a 5-carbon sugar, with the accompanying production of another mol of NADPH.

The ribose-5-phosphate produced can now be utilized in the synthesis of nucleotides for incorporation into nucleic acids. The reaction proceeds through an enediol (C=C double bond and two hydroxyl groups) intermediate, as the enzyme takes advantage of the dissociability of the hydrogen on the terminal hydroxyl group to create an oxyanion and move the C=O double bond to the terminal carbon, producing the aldehyde and reducing the ketone to an alcohol.

It is important to control ribose synthesis, a mechanism exists to remove this sugar when it is in excess, by converting it into glycolytic intermediates. A series of three enzymatic steps are carried out, transferring two- and three-carbon fragments from one sugar to another, and all of these steps are similar in mechanism to an aldol condensation (recall that aldolase, the enzyme in glycolysis, which fragments the six-carbon, bisphosphorylated sugar fructose 1,6-bisphosphate into two phosphorylated three-carbon fragments, breaks the carbon-carbon bond through the reverse mechanism of aldol condensation. Transketolase, is the enzyme which transfers a two-carbon fragment terminating on the interior side in a carbonyl, and transaldolase, transfers a three-carbon fragment terminating on the central side in a hydroxymethylene group.

The convertion of ribose 5-phosphate to glycolytic intermediates is catalysed by transketolase, by the transfer of the 1- and 2-carbons from xylulose 5-phosphate to the 1-carbon of ribose 5-phosphate. This leaves the last three carbons from xylulose-5-phosphate as glyceraldehyde 3-phosphate, the first three-carbon fragment encountered in glycolysis, and sedoheptulose 7-phosphate, produced from the ribose 5-phosphate, which is a seven-carbon sugar. By inverting the cofigration at carbon -3, xylulose 5-phosphate can be produced from ribulose 5-phosphate. This reaction is carried out by the enzyme phosphopentose epimerase, and is freely reversible. Thus, in the first reaction, converting ribose-5-phosphate to glycolytic intermediates, both ribose-

5-phosphate and ribulose 5-phosphate (the latter in the form of xylulose 5-phosphate) are being degraded to other species, and eventually carried off in glycolysis.

Transaldolase mediates the second reaction which leads from the intermediates in the pentose phosphate pathway to glycolytic. This enzyme transfers a three-carbon fragment (carbons 1, 2 and 3) from the sedoheptulose 7-phosphate just formed in the first reaction to the glyceraldehyde 3-phosphate just formed in the first reaction, yielding a four-carbon fragment, erythrose 4-phosphate, and a six-carbon fragment, fructose 6-phosphate. The fructose 6-phosphate is now liberated to enter the glycolytic pathway.

Transketolase catalyses the final reaction,s linking the pentose pathway to the glyolytic pathway. In this reaction, another molecule of xylulose 5-phosphate is cleaved, and the two-carbon fragment consisting of carbons 1 and 2 is transferred to the molecule of erythrose 4-phosphate just formed in the transaldolase reaction, generating a molecule of glyceraldehyde 3-phosphate and another molecule of fructose 6-phosphate. Both of these products are capable of entering glycolysis directly, and so there are no leftover fragments formed in this overall convertion. Because another molecule of xylulose 5-phosphate has entered the reaction, the overall conversion consists of two molecules of xylulose 5-phosphate and one molecule of ribose 5-phosphate going to two molecules of fructose 6-phosphate and one molecule of glyceraldehyde 3-phosphate, and so the net reaction is the removal to glycolysis of three molecules of ribose 5-phosphate.

For different purpose NADPH and ribose 5-phosphate produced by the pentose phosphate pathway it is sometimes necessary to produce them in differing proportions. Therefore, the cell has different modes in which the pentose phosphate pathway can function. In the case, where much more ribose-5-phosphate is required than NADPH, the ribose 5-phosphate may be produced from glyceraldehyde 3-phosphate and fructose 6-phosphate through the reversal of the transaldolase and transketolase reactions. This allows the cell's $NADP^+$ supply to remain relatively unaffected.

When both NADPH and ribose 5-phosphate are desired in large amounts, the principal reaction used by the cell to generate them is the conversion of glucose-6-phosphate to ribose 5-phosphate, with the liberation of two molecules of NADPH for each molecule of glucose 6-phosphate converted.

The important role of the NADPH produced in the pentose phosphate pathway is the maintenance of the glutathione in its reduced state through the action of gluthathione reductase. In terms reduced glutathione plays an important role in protection of the cell against oxidative damage.
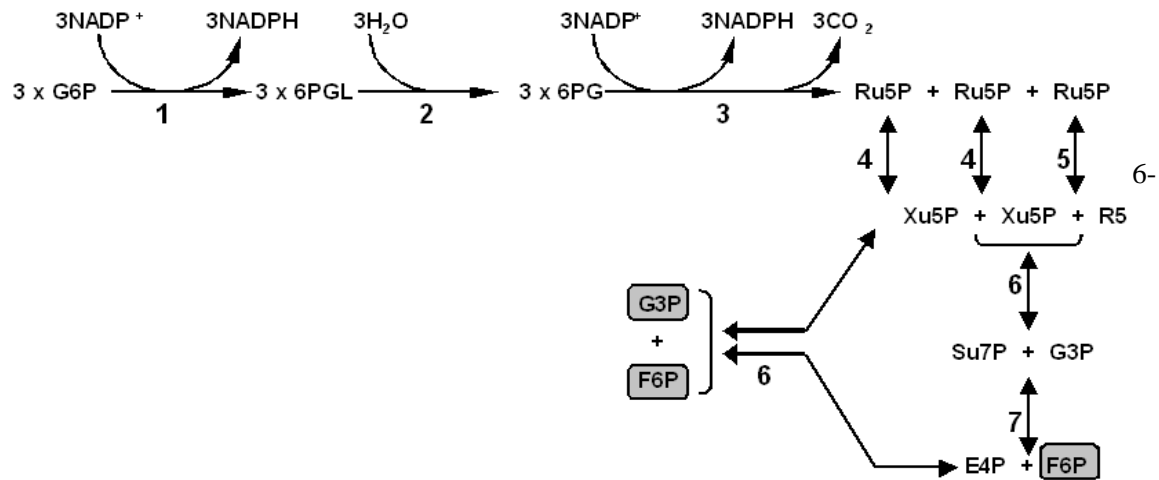
4. ribulose 5-phosphate → ribose 5-phosphate (phosphopentose isomerase)

5. ribulose 5-phosphate → xylulose 5-phosphate (phosphopentose epimerase)

6. xylulose 5-phosphate + ribose 5-phosphate → glyceraldehyde 3-phosphate +

   Sedoheptulose 7-phosphate (transketolase)

7. Sedoheptulose 7-phosphate + glyceraldehyde 3-phosphate → erythrose 4-phosphate +

   fructose 6-phosphate (transaldolase)

6. Fructose 6-phosphate + erythrose 4-phosphate → glyceraldehyde 3-phosphate +

   Glucose 6-phosphate (transketolase)

1.5.3.1 Xylulose 5-phosphate

Xylulose 5-phosphate is a product of pentose phosphate pathway. It has been reported to activate PP2A allosterically (Nishimura et al.; 1995). In the liver xylulose 5-phosphate regulates the bifunctional enzyme through allosteric activation of protein phosphatase (Pelech *et al.*) The activation of PP2A by Xu 5-P in rat liver, represents one of only two examples of metabolite (or small *M*r compounds) that allosterically activate PP2A. The other is the activation of a heterotrimeric form of PP2A of rat T9 glioma cells by ceramide (Dobrowsky and Hunnun, 1992) (Dobrowsky *et al.,* 1993). However, a more recent report (Law and Rossle, 1995) indicated that even the catalytic subunit is activated by ceramide and this effect lacks substrate specificity. This is in contrast to the Xu 5-P-activated PP2A, because activation by Xu 5-P shows the substrate specificity, thus reflecting the differences in the activation mechanism. Since PP2A is not only the most abundant form of protein phosphatase, especially in cytoplasm, but also shows broad substrate specificities, it is possible that other PP2As may be regulated by specific metabolites.

1.5.3.2 Glucose 6 - phosphate dehydrogenase

Glucose 6-phosphate dehydrogenase (G6PD) functions as the first and rate-limiting enzyme in the pentose phosphate pathway (PPP), responsible for the generation of NADPH in a reaction coupled to oxidation of glucose 6- phosphate and the de novo production of cellular ribose 5-phosphate. The activity of G6PD is rapidly increased in response to cellular oxidative stress, by the translocation of G6PH to the cell membrane. Inhibition of G6PD leads to the depletion of the cytosolic reduced glutathione (GSH) (Eaton et al.; 1999). In cardiomyocytes, GSH resides in two separate compartments, mitochondria and cytosol. When its level is depleted, the subsequent result in cardiomyocytes is contractile dysfunction through the dysregulation of calcium homeostasis. G6PDH is a critical cytosolic antioxidant enzyme, essential for the maintenance of cytosolic redox status in adult cardiomyocytes (Mohit *et al*.; 2003).

In the isolated perfused rabbit heart ischemia induced a rapid decline of contractility, associated with a reduction in the content of tissue GSH with no significant changes in GSSG. Reperfusion induced a small recovery of contractility, a substantial release of total glutathione in the cytosol and a further decrease in the content of tissue GSH, with a significant increase in tissue GSSG. Glutathione reductase and glutathione peroxidase activities were unaffected by ischemia and reperfusion. This study suggests a possible role for glutathione in the determination of functional damage induced by myocardial ischemia and reperfusion (Curello *et al.;* 1987).

1.5.3.3 Transketolase

Transketolase is a dimeric enzyme with the active sites located at the interface between the two identical subunits. The cofactor, vitamin B1 derived thiamine pyrophosphate, is bound at the interface between the two subunits. The enzyme subunit is composed of three domains of the alpha/beta type. The diphosphate moiety of thiamine diphosphate is bound to the enzyme at the carboxyl end of the parallel beta-sheet of the N-terminal domain and interacts with the protein through a Ca^{2+} ion. The thiazolium ring interacts with residues from both subunits, whereas the pyrimidine ring is buried in a hydrophobic pocket of the enzyme, formed by the loops at the carboxyl end of the beta-sheet in the middle domain of the second subunit. Structural analysis identifies the amino acids that are critical for cofactor binding and provides mechanistic insights into thiamine catalysis.

. 1.5.3.4 D- Ribose and Xylitol

D-ribose is ubiquitous in living matter; it is ingested through our diet. Recent research suggests that supraphysiological amounts of this sugar may have cardioprotective effects, particularly for the ischemic heart. Ribose has been shown greatly to increase ATP recovery in situations such as postischemia, when all of the ATP has been depleted by catabolism. Furthermore, metabolic studies have reported that both sugars and alcohols (ribose and xylitol) can support energy

metabolism, presumably after conversion to substrates for glycolysis. In hearts perfused with ribose, xylitol or no substrate, the rate pressure product and the stroke volume rapidly declined after an initial brief stable period corresponding to glycogen depletion (Mahoney *et al.;* 1989). By contrast, either glucose or pyruvate supported steady levels of ATP and myocardial oxygen consumption, maintained the energy charge, and supported the stroke volume, rate pressure product, and cardiac work. Thus, although the heart is able to metabolize ribose and xylitol through the hexose monophosphate pathway, the rate of utilization through glycolysis and presumably the TCA cycle is insufficient for these compounds to serve as exclusive substrates for the isolated working heart (Mahoney *et al.;* 1989).

15.3.4 Thiamine

Thiamine plays an essential role in the metabolism of glucose. Previous studies have demonstrated that thiamine has a cytoprotective effect against ischemic damage to the heart and to heat shock protein 70 (Hsp70). This thiamine effect also protects cardiac cells from lethal ischemia/hypoxia (Jaattla, 1999; Latchman, 2001). Thiamine (Vitamin B1) is also a clinically important factor in heart function, and its deficiency leads to heart failure (Ozawa *et al.;* 2001). The accumulation of glycerol 3-phosphate, and the increased formation of the potent glycating agent, methylglyoxal, in intracellular hyperglycaemia, is implicated in the development of diabetic complications. A strategy for countering this is to stimulate the anaerobic pentose phosphate pathway of glucose metabolism by maximising transketolase activity by thiamine supplementation. This leads to the consumption of glyceraldehyde-3-phosphate, an increase in the formation of ribose 5-phosphate (R 5-P). Increased R 5-P may stimulate de novo purine synthesis by increasing the availability of ribosylpyrophosphate. R 5-P is a glycating agent, but is much less reactive than methylglyoxal and is present at a low concentration ($20\mu M$) in cells.

1.5.4 Hexosamine biosynthetic pathway

Glucose phosphorylated by hexokinase to glucose 6-phosphate is converted to fructose 6-phosphate in the next step of glycolysis. Fructose 6-phosphate can be converted into

UDP-N-acetylglucosamine (UDPGlcNac) by glutamine: fructose 6-phosphate-amidotransferase (GFAT), the first and rate-limiting enzyme of the hexosamine biosynthetic pathway. UDPGlcNac serves as the substrate for the O-GlcNacylation of proteins by the enzyme O-GlcNac transferase (OGT). O-GlcNacylation has been shown to compete for phosphorylation sites of signalling proteins and transcription factors, thereby modifying their activity (McClain *et al*.; 2002).

1.5.4.1 Glutamine fructose 6-amidotransferase

Glutamine fructose 6- phosphate amidotransferase (GFAT) is the rate limiting enzyme in hexosamine biosynthesis pathway, an important pathway for cellular glucose sensing. It also has been implicated in insulin resistance. GFAT has two isoforms GFAT1 and GFAT2. GFAT1 is ubiquitous, whereas GFAT2 is expressed mainly in the central nervous system (Deharen *et al*.; 2001). The mouse GFAT2 cDNA was cloned, and the protein was expressed with either an N-terminal glutathione *S*-transferase or His tag (Deharen *et al*.; 2001). The purified protein expressed in mammalian cells had GFAT activity. The K_m values for the two substrates of reaction, fructose 6-phosphate and glutamine, were determined to be 0.8 mM for fructose 6-phosphate and 1.2 mM for glutamine, which are within the ranges determined for GFAT1. The protein sequence around the serine 202 of GFAT2 was conserved to the serine 205 of GFAT1, whereas the serine at 235 in GFAT1 was not present in GFAT2. Previously it was shown that phosphorylation of serine 205 in GFAT1 by the catalytic subunit of cAMP-dependent protein kinase (PKA) inhibits its activity. Like GFAT1, GFAT2 was phosphorylated by PKA, but GFAT2 activity increased approximately 2.2-fold by this modification. When serine 202 of GFAT2 was mutated to an alanine, the enzyme not only became resistant to phosphorylation, but the increase

in activity in response to PKA also was blocked. The phosphorylation of serine 202 was necessary and sufficient for these alterations by PKA. GFAT2 was modestly inhibited (15%) by UDP-GlcNAc but not through detectable *O*-glycosylation. GFAT2 is, therefore, an isoenzyme of GFAT1, but its regulation by cAMP is the opposite, allowing differential regulation of the hexosamine pathway in specialized tissues (Hue *et al*.; 2004). Recent study has proved that phosphorylation of GFAT1 at Ser243 by AMPK has an important role in the regulation of the GFAT1 enzymatic activity (Yonezawa   *et al*.; 2009)

1.5.5 Glycogen

Glycogen is a high-molecular-weight polysaccharide that serves as a repository for glucose units for utilization in times of metabolic need. The glycogen pool in the heart is relatively small ($\sim$30 µmol/g wet wt compared with $\sim$150 µmol/g wet wt in skeletal muscle) (Arad *et al.;* 1994, Opie *et al*.; 1991) and has a relatively rapid turnover, despite stable tissue concentrations (Henning *et al*.; 1996). An alternative source of glucose 6-phosphate for the heart is the intracellular glycogen store. Glycogen concentrations are increased by a high supply of exogenous substrate and/or hyperinsulinemia (Kruszynska *et al*.; 1991, Laughlin *et al*.; 1994). Glycogenolysis is activated by adrenergic stimulation (e.g., increases in cAMP and Ca^{2+}), a fall in the tissue content of ATP, and a rise in inorganic phosphate, that occurs during ischemia or intense exercise. (Hue *et al*.; 1995). Recently, there has been considerable focus on the role of AMPK in regulating the glycogen content of the heart (Arad *et al*.; 2003). Constitutively inactive AMPK, due to a mutation in a regulatory subunit of the enzyme, was recently shown to be linked with increased glycogen content and hypertrophic cardiomyopathy (Arad *et al*.; 2003, Seidman *et al*.; 2003). In contrast acute activation of AMPK has been shown to activate glycogenolysis (Longnus 2003, Polekhina *et al*.; 2003). Clinically, patients with a mutation in the gamma-2 regulatory subunit of AMPK have Wolff-Parkinson-White syndrome and conduction system disease in the absence of cardiac

hypertrophy (Gollob *et al*.; 2003, Gollob *et al*.; 2001), although the cellular mechanisms linking abnormal AMPK activity and the electrophysiological abnormalities remain unclear.

Mammals express two isoforms of glycogen synthase, encoded by the *GYS1* and *GYS2* genes. *GYS1*, encoding the muscle isoform of glycogen synthase, is expressed in skeletal muscle, cardiac muscle, adipose tissue, kidney and brain (Kaslow *et al*.; 1984). *GYS2* expression has been found only in the liver (Tan *al et*.; 1985). Many cells can synthesize glycogen but, in absolute amounts, the major stores are in the liver and skeletal muscle. Although its essential role as an energy reserve is common to all cells, there are differences in glycogen function between tissues. Liver glycogen contributes primarily to blood glucose homeostasis, being synthesized during periods of nutritional sufficiency, and subsequently converted to glucose, which is released into the bloodstream to counteract hypoglycemia (Roche *et al*.; 2001).

 In the fed state, glycogen is also synthesized in skeletal muscle, where it functions as an energy reserve for fuel contraction (Roch *et al*.; 2001). Glycogen provides a part of the glucose utilized by the aerobic working adult heart (Depre *et al*.; 1999) and is preferentially oxidized compared to exogenous glucose (Goodwin *et al*.; 1996). Although long-chain fatty acids are normally the major substrates for the adult heart, when blood glucose and insulin concentrations are high, such as after a meal, glucose becomes a more prominent energy source (Nakal *et al*.; 1994 (Henning *et al*.;1996). Various stresses also trigger a greater reliance on glycogen. When the workload of the heart is increased, after stimulation by adrenaline (Nakal *et al*.; 1994; Taegtmeyer *et al*.; 1998) and/or by increased exercise (Goodwin *et al*.; 1996; Henning *et al*.; 1996), glycogen provides a readily reasonable source of additional energy. In ischemia, glucose assumes a more important role as a fuel for the heart, since fatty acid oxidation is limited and glycogen supplies glucose for anaerobic glycolysis (Depre   *et al*.; 1999).

1.6 Lipid fuels

Fatty acids are an important source of energy for many organisms. Excess glucose carbon can be stored efficiently as fat. Triacylglycerols (TAG) yield more than twice as much energy for the same mass as do carbohydrates or proteins. All cell membranes are composed of phospholipids, each of which contain fatty acids. Fatty acids are also used for protein modification. The metabolism of fatty acids, therefore, consists of catabolic processes which generate energy and primary metabolites from fatty acids, and anabolic processes which create biologically important molecules from fatty acids and other dietary carbon sources.

Cardiac myocytes have a very low capacity for *de novo* fatty acid synthesis and so rely on supply of fatty acids from the circulation. These fatty acids may be oxidized to provide energy or esterfied to form an endogenous TAG store. Fatty acids are present in the blood as either unesterified molecules, or incorporated into phospholipids, cholesteryl ester or as acylglycerols. The solubility of free fatty acids in the plasma is increased by the formation of a fatty acid – albumin complex. Over 90% of plasma fatty acids are present as esters, which are associated with proteins other than albumin to increase their solubility (Fredrickson and Gordon, 1958). Blood-borne TAG is an important source of myocardial fatty acids. These hydrophobic TAG molecules form the core of lipoprotein articles, such as Very Low Density Lipoproteins (VLDL) and chylomicrons, which are surrounded by a hydrophilic layer composed of phospholipids, cholesterol and apoproteins.

The 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 cannot diffuse between endothelial cells, so the transport of fatty acids through the endothelium in complex with albumin is unlikely (Den *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 the direct transfer of

the fatty acids (Bassingthwaighte *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 through the action of lipoprotein lipase (Cryer *et al*.; 1989). The rate of hydrolysis of the circulating TAG is largely dependent on TAG concentration in the circulation and the amount of LPL at the endothelials luminal surface. The full activity of LPL also depends on the presence of apoprotein CII which is found on the surface of lipoproteins. The accumulation of free fatty acids and monoacylglycerols inhibit the hydrolytic activity of LPL (Crona *et al.;* 1987). The short-term regulation of LPL activity is mainly due to alterations in the rates of secretion and loss of enzyme from the endothelial cell surface. The long-term regulation of LPL activity is likely to occur as a result of alterations in the rates of enzyme synthesis and degradation within the myocytes. Rats treated with corticosteroids, adrenocorticotrophic hormone (ACTH) and thyroxine showed a long-term stimulation of total activity. Diabetes has also been shown to increase LPL activity in the heart (Stam *et al*.; 1984). The increase in LPL activity which occurs during fat feeding is likely to be due to glucocorticoids (Pedersen *et al*.; 1981). Unlike skeletal muscle, where it causes a decrease in LPL synthesis, in the heart, insulin does not alter LPL activity (Stam *et al.;* 1984).

Hormone-Sensitive Lipase (HSL) is the enzyme responsble for hormonally stimulated lipolysis. It has been shown to be present in tissue other than adipocytes, including heart and skeletal muscle (Small *et al*.; 1989). HSL 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 idetified in rat HSL as the position which is phosphorylated by PKA in response to lipolytic stimulation (Garton *et al*.; 1988). A second phosphorylation site is located at residue 565 in the rat sequence (Stralfors   *et al.;* 1984). A number of protein kinases act at this site, including AMPK (Garton *et al.;* 1989). The activity of HSL can also be regulated by protein phosphatases, such as protein phosphatase 1, 2A and 2C.

When blood sugar is low, glucagon signals the adipocytes to activate hormone sensitive lipase, and to convert TAG into free fatty acids. These have very low solubility in the blood, typically about 1 μM. However, the most abundant protein in the blood, serum albumin, binds free fatty acids, increasing their effective solubility to ~ 1 mM. Thus, serum albumin transports fatty acids to organs such as the muscle, heart and liver for oxidation when the blood sugar is low. Fatty acid degradation is the process whereby fatty acids are broken down, resulting in a release of energy.

1.6.1 Fatty acids

Fatty acids are carboxylic acids with long hydrocarbon chains. The hydrocarbon chain length may vary from 10-30 carbons (commonly 12-18 in mammals). In acids with only a few carbons, the acid functional group dominates and gives the whole molecule a polar character. However, in long chain fatty acids, the non-polar hydrocarbon chain gives the molecule a non-polar character.

1.6.1.1 Fatty acid oxidation

Fatty acid oxidation is an important metabolic process carried out in the mitochondria. Heart fatty acids are the central source of energy under a variety of conditions (Neely and Morgan 1974). The pathway and enzymology of mitochondrial fatty acid oxidation have been reviewed (Schulz *et at*.; 1991). The first step on this pathway is the cellular uptake of free fatty acids. The mechanism of this process remains a matter of dispute, with some studies concluding that plasma free fatty acids enter the cells by a carrier mediated uptake, and others claiming that the uptake occurs due to the spontaneous diffusion of fatty acids across the cellular membrane. In the cytosol, free fatty acids are associated with fatty acid-binding proteins (FABPs). Because all of the tissues active in fatty acid metabolism contain at least one of these low-molecular weight proteins, they have been proposed to function in fatty acid uptake and intracellular transport. Fatty acids are trapped within the cells due to their conversion to CoA thioesters catalyzed by acyl-CoA synthetases. The long-chain acyl-CoA synthetases are membrane-bound enzymes that

are connected with the outer mitochondrial membrane as well as with the endoplasmic reticulum and peroxisomes.

Medium-chain acyl-CoA synthetase is present in the mitochondrial matrix, whereas short-chain acyl-CoA synthetase is located both in the cytosol and in the mitochondrial matrix. Because the inner mitochondrial membrane is a barrier to free CoA (CoASH) and acyl-CoA, fatty acyl residues are carried across this membrane as carnitine esters. Carnitine palmitoyltransferase I (CPTI), which is located at the outer mitochondrial membrane, transfers fatty acyl residues from CoA to L-carnitine. The resulting fatty acyl carnitines pass through the inner mitochondrial membrane via carnitine: acylcarnitine translocase by 1:1 exchange for carnitine or other acylcarnitines, including acetylcarnitine.Carnitine palmitoyltransferase II (CPTII) associated with the mitochondrial inner membrane catalyzes the transfer of fatty acyl residues back from carnitine to acyl- CoA in the mitchondrial matrix.

 Medium-chain fatty acids, in contrast to long-chain ones, can pass directly through the inner mitochondrial membrane and can be activated in the matrix by medium-chain acyl-CoA synthetase. Acyl-CoA, created in the matrix from long-chain acylcarnitines or medium-chain fatty acids, are substrates for the β-oxidation spiral that yields acetyl-CoA, NADH, and $FADH_2$. The latter two compounds are reoxidized by the mitochondrial electron transport chain, whereas acetyl-CoA is oxidized to CO_2 by the tricarboxylic acid cycle which regenerates CoASH. The enzymes catalyzing the reactions of the β-oxidation spiral are acyl- CoA dehydrogenase, enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase. At least two isoforms of each of the four enzymes are present in mitochondria. The isozymes which have different but overlapping chain length specificities cooperate during the complete degradation of fatty acids to acetyl-CoA. Because the intermediates of β-oxidation do not build up to any significant extent and because the ability of this process dramatically declines when the mitochondria are disrupted, it has been speculated that the enzymes of β-oxidation exist as

organized complexes in involvement with the inner mitochondrial membrane. This situation makes it impossible to study the regulation of β-oxidation with soluble mitochondrial extracts.

The rate of fatty acid β-oxidation in the heart depends on the availability of exogenous fatty acids and on the utilization of energy by the tissue (Neely and Morgan 1974). Because the release of free fatty acids from the adipose tissue is regulated via hormone-sensitive lipase (Stralfors *et al*), hormones like glucagon, adrenaline and insulin indirectly influence myocardial β-oxidation by controlling the availability of substrate.

1.6.1.2 Ketone Bodies

Ketone bodies are molecules that the body makes when there is insufficient insulin in the blood and it must break down fat instead of glucose for energy. The ketone bodies, acetoacetate, and β-hydroxybutyrate are acidic and are harmful when in excess. Acetoacetate and β-hydroxybutyrate are formed from fatty acids by the liver, and are transported in the bloodstream for use as metabolic fuel by other tissues. Acetoacetate is chemically unstable and breaks down to acetone, which is poorly metabolized, and is excreted in the urine and on the breath.

The heart extracts and oxidizes ketone bodies (β-hydroxybutyrate and acetoacetate) in a concentration-dependent way (Chen *et al*.; 1984). Plasma ketone bodies are produced from fatty acids in the liver, and the arterial plasma concentration in the fed state is normally very low; thus they are normally a minor substrate for the myocardium. Through starvation or poorly-controlled diabetes, plasma ketone body concentrations rise as result of low insulin and high fatty acids, and they become a significant substrate for the myocardium (Avogaro *et al*.; 1990).

Cardiac fatty acid oxidation is inhibited by ketone bodies oxidation (Forsey *et al*.; 1987), Lammerant *et al*.; 1985). The diabetic myocardium has a relatively low rate of fatty acid uptake and a high rate of β-hydroxybutyrate uptake (Hall *et al*.; 1996), signifying that, in diabetic

patients, raised plasma ketone concentrations can act to inhibit fatty acid uptake and oxidation. Clinical studies demonstrate that high fatty acids result in an increase in plasma ketone body concentration that appears to be secondary to elevated fatty acid levels (Lommi *et al.;* 1997); however, data on myocardial ketone body metabolism have not been reported from animals or patients with high fatty acids. The biochemical mechanisms responsible for the inhibition of fatty acid β-oxidation by ketone bodies are not well understood, but do not appear to be related to changes in malonyl-CoA or the acetyl-CoA/CoA ratio (Stanley *et al*.; 2003).

Raising the rates of β-hydroxybutyrate and acetoacetate oxidation could inhibit fatty acid β-oxidation by increasing the intramitochondrial ratio of NADH to NAD^+ which would inhibit the ketoacyl-CoA dehydrogenase step of the fatty acid β-oxidation spiral (Kunau *et al*.; 1995). Vusse and workers recently showed that palmitate oxidation was considerably enhanced in isolated cardiomyocytes from streptozotocin diabetic rats in the absence of acetoacetate; however, when measurements were made in the presence of ketone bodies, the rate of palmitate oxidation was not influenced by diabetes (Vusse *et al*.; 2003). They also noted a greater fatty acid uptake in the myocytes of diabetic rats and suggested that ketone-induced impairment of fatty acid oxidation might be accountable for the greater TAG storage in the heart with diabetes (Denton and Randle 1967).

1.6.1.3 Acetyl-CoA Carboxylase

.Acetyl-CoA carboxylase (ACC) is a biotin-dependent enzyme that catalyzes the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA through its two catalytic activities: biotin carboxylase and carboxyltransferase.

Mammalian ACC is expressed as two proteins, termed ACC265,000, ACC-1 or ACC-α and ACC 280,000, ACC-2 or ACC-β. ACC- α is thought to function more, but not exclusively, as a

provider of malonyl-CoA for lipogenesis, wherease ACC- β is thought to function primarily as source of malonyl-CoA to regulate CPT1 and control mitochondrial β-oxidation. ACC- α is essential for embryonic development in mice (Abu-Elheiga *et al*.; 2005). It is the most abundant form of ACC protein in lipogenic tissue such as liver, white adipose tissue, and mammary gland (Bianchi A et al.; 1990) but it also found in other tissues such as heart (Saddik *et al*.; 1993) and pancreatic islet (Louis *et al.;* 1988). Knockout of the ACC β gene results in a nonlethal phenotype in which animal have increased β-oxidation in liver and muscle, are lean, and hyperphagic. These animals are resistant to obesity and diet-induced diabetes. In muscle, they have a decreased malonyl-CoA content whereas hepatic malonyl-CoA is unchanged (Abu-Elheiga *et al*.; 2002). Compared with ACC-α ACC- β has approximately 140 extra amino acid residues as its N-terminus, which contains a motif that allows targeting and /or binding to the mitochondrial outer membrane. It suggested that this could permit control of the malonyl-CoA concentration near the malonyl- CoA binding site of CPT1 (Kim *et al*.; 1997).

Regulation of ACC activity can be 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 and Guy 1980). However changes in cellular citrate concentration do not always correspond to change in ACC activity and sensitivity of ACC to citrate.

Long –chain fatty acyl CoA thioesters allosterically inhibit ACC. ACC α contains several potential serine phosphorylation sites. Those phosphorylated by protein kinase A (PKA) (Ser 1200) and AMPK(Ser79) produce inactivation of ACC in vivo. AMPK and PKA both increased the K_a for citrate. Nutritional and hormonal control of ACCα gene expression occurs. ACC expression is decreased in liver and white adipose tissue by fasting and diabetes and is restored by reefeding but these manipulations have no appreciable effect on ACC expression in muscle tissue (Barber *et al.;* 2005).

1.7 **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 reaction generates 2 molecules of CO_2 3NADH 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 O2 is not directly involved in the enzymatic reaction, 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 regulator 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 *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 heart are perfused with octanoate ,acetyl-CoA for citrate synthase is derived from β-oxidation, a situation which favours pyruvate carboxylation (Comte *et al.;* 1997) Under these conditions the high availability of both acetyl- CoA and oxloacetate 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 intrmitochondrial NADH decreases there is increased conversion of malate to oxloacetate and greater flux through the citrate synthase reaction n (Bowman 1966). Aspartate transamination is also an important mechanism for the generation of oxaloacetate.

Two other mechanisms are involved in regulating citric acid cycle activity. First the redox state of FAD and NAD^+ and secondly the allosteric control of citrate synthase and dehydrogenase reactions (LaNoue *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 K_m for acetyl-CoA, and also inhibit the isocitrate dehydrogenase

reaction. Isocitrate dehydrogenase is inhibited by NADH and stimulated by ADP which decrease the Km for reaction substrates isocitrate and NAD^+. α-ketogluarate 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 et al.; 1990).

1.8 Oxidative phosphorylation

In eukaryotes, oxidative phosphorylation occurs in the mitochondrial cristae. It comprises the electron transport chain that establishes a proton gradient (chemiosmotic potential) across the inner membrane by oxidizing the NADH and FADH produced from the Krebs cycle and β-oxidation and other processes. ATP is synthesised by the ATP synthase enzyme when the chemiosmotic proton gradient is used to drive the phosphorylation of ADP. The electrons are finally transferred to exogenous oxygen, with the addition of two protons to form water**.**

1.9 Hormones

1.9.1 Insulin

Insulin that is produced in the Islets of Langerhans in the pancreas is a polypeptide hormone that regulates carbohydrate and lipid metabolism. 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 *et al.;* 1991) and adipose tissue (Lawrence *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 *et al*.; 2004), an effect which was overcome by increasing the concentration of palmitate to 1.2mM (with 20 mM glucose). Insulin has also been shown to enhance glycogen synthesis and inhibit glycogenolysis in perfused rat hearts (Goodwin *et al*.; 1995). These effects are due to insulin modulation of the activities of glycogen synthase and phosphorylase.

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 hormonal stimulation of glucose uptake these two dephosphorylation events explain the increased tissue glycogen content of tissues exposed to insulin.

In addition to its role in regulating glucose metabolism, insulin stimulates lipogenesis, diminishes lipolysis, and increases amino acid transport into cells. Insulin also modulates transcription, altering the cell content of numerous mRNAs. It stimulates growth, DNA synthesis, and cell replication, effects that it holds in common with the insulin-like growth factors (IGFs) and

relaxin. Insulin has been shown to inhibit AMPK activity in the heart (Gamble and Lopaschuk 1997; Clark *et al*.; 2004). This effect was shown to be independent of changes in the AMP/ATP ratio (Beauloye *et al*.; 2001). Moreover, insulin markedly decreased the extent of the AMPK activation and Thr172 phosphorylation increased by ischemia or anoxia by a mechanism which was again independent of the AMP/ATP and Cr/CrP (Soltys *et al*.; 2006).

1.9.1.1 Insulin Receptor

The insulin receptor is a transmembrane glycoprotein with four subunits, two α2 and two β2. The extracellular α-subunit contains the insulin-binding site, and the intracellular portion of β-subunit contains a tyrosine kinase domain. The insulin receptor gene is mapped to the short arm of human chromosome 19, and it contains 22 exons (Ebina *et al.;*1985; Ullrich *et al*.; 1985). The insulin receptor is synthesized as a single polypeptide precursor. Following its transport to the plasma membrane, two monomers aggregate to form a dimeric proreceptor which is then cleaved at the four-amino-acid site (RKRR) to produce the heterotetrameric insulin receptor (Alarcon *et al*.; 1995). The insulin receptor is expressed ubiquitously. The highest levels are found in major insulin target tissues (e.g., liver, skeletal muscle and adipose tissue), and lower levels are found in relatively insulin-unresponsive tissues (e.g., circulating erythrocytes and brain) (Bruning *et al.;* 2000).

The insulin receptor gene is alternatively spliced to yield two isoforms that differ by the absence or presence of exon 11. In the carboxyl terminus of α subunit, isoform A (IR-A) lacks the amino acids encoded by exon 11 that are present in isoform B (IR-B). The functional difference between IR-A and IR-B is small, except that IR-A has a higher affinity for insulin and IGF-1 (Amaguchi *et al.;* 1995) and mediates an accelerated rate of receptor mediated insulin internalization (Vogt *et al.;* 2003).

 In normal Sprague-Dawley rats, IR-B is the predominant isoform of insulin receptor expressed in liver, adipose tissue, and kidney, whereas IR-A is predominant in muscle, hypothalamus and cerebral cortex (Luo *et al*.; 1998). A similar distribution pattern was observed in cell lines of

corresponding tissue origins (Moller *et al.;* 1989). The biological significance of having two isoforms of IR is still unclear. Isoform switching of the insulin receptor has been documented during development, with a higher percentage of IR-A in early stages, and a higher expression of IR-B in the adult (White, *et al.*; 2002). A similar shift in expression patterns of the two isoforms has been reported in diabetes; however, this issue remains inconclusive due to contradictory evidence (Sbraccia *et al.;* 1998).

1.9.1.2   Insulin Receptor Substrate-1

IRS-1 acts as a docking protein that mediates multiple interactions among other proteins in cells. It plays a key role in transmitting signals from the insulin and insulin-like growth factor-1 (IGF-1) receptors to intracellular pathways such as PI3K / *Akt* and Erk / MAP kinase pathways. The insulin receptor is composed of two extracellular α subunits and two transmembrane β subunits linked together by disulphide bonds. Binding of insulin to the α subunit induces a conformational change resulting in the autophosphorylation of a number of tyrosine residues present in the β subunit (Obberghen *et al.;* 2001). These residues are recognised by phosphotyrosine-binding (PTB) domains of adaptor proteins such as members of the insulin receptor substrate family (IRS) (Saltiel and Kahn 2001). Receptor activation leads to the phosphorylation of key tyrosine residues on IRS proteins, some of which are recognised by the Src homology 2 (SH2) domain of the p85 regulatory subunit of PI 3-kinase (a lipid kinase).

IRS-1 plays an important biological function for both metabolic and mitogenic (growth promoting) pathways. Mice deficent in IRS1 have only a mild diabetic phenotype, but pronounced growth restriction. IRS-1 knockout mice only reach 50% of the weight of normal mice. IRS-1 may also play a role in cancer, as it has been shown that transgenic mice overexpressing IRS-1 develop breast cancer (Dearth *et al.;* 2006). The cellular protein levels of

IRS-1 are regulated by the Cullin7 E3 ubiquin ligase, which targets IRS-1 for ubiquitin mediated degradation by the proteasome (Sarikas et *al.;* 2008).

1.9.1.3 Phosphoinositide 3-kinases

PI3Ks are lipid kinases that phosphorylate the inositol ring of phosphatidylinositol (PtdIns) and various phosphatidylinositol phosphates at the 3 position. PI3Ks are grouped into three classes; classes I, II, and III. *In vivo*, class I PI3Ks predominantly phosphorylate PtdIns (4, 5) P2 to generate PtdIns (3, 4, 5)P_3, whereas class II and III mainly phosphorylate PtdIns to generate PtdIns (3) P (Oudit *et al* 2004).

Class I

 The class I PI3Ks are heterodimeric enzymes consisting of catalytic and regulatory adaptor subunits, and they are further divided into class IA and IB. There are three isoforms for class IA catalytic subunits; p110α, p110β, and p110δ, and three isoforms for the adaptor subunit; p85α, p85β, and p55γ. The class IB PI3K (PI3Kγ) is composed of the catalytic subunit p110γ tightly bound to the regulatory subunit p101. The adaptor subunits for the class IA PI3Ks contain a SH2 domain as well as a p110 binding domain, and they recruit the catalytic subunit to the plasma membrane upon tyrosine phosphorylation following, for example, receptor tyrosine kinase activation by insulin. In contrast, the regulatory subunit of PI3Kγ lacks the SH2 domain. Instead, both the catalytic and regulatory subunits of PI3Kγ contain the binding sites for $G_{\beta\gamma}$ (Wymann *et al* 2003). PI3Kγ is recruited and activated by $G_{\beta\gamma}$ following activation of GPCRs (Stephens *et al* 1997; Alloatti *et al* 2004; Naga Prasad *et al* 2000). Both class IA PI3Ks and PI3Kγ catalytic subunits also contain the C2 domain involved in phospholipid binding, the PIK domain involved

in protein-protein interaction, and the Ras binding domain. Ras-GTP binding enhances the catalytic activity of both class IA PI3K and PI3Kγ (Wymann *et al* 2003).

Class II and Class III

Class I PI3K is differentiated from class PI3KS II and III by its function and structure. Class II unlike class I and III has no regulatory protein. It has three catalytic isoform (C2α, C2β, and C2γ). These enzymes catalyze the formation of PI (3)P from PI and may also produce PI(3,4)P$_2$ from PI(4)P). C2α and C2β are widely expressed through the body, however expression of C2γ is limited to hepatocytes. The distinct feature of Class II PI3Ks is the C-terminal C2 domain. This domain lacks vital Asp residues to coordinate binding of Ca^{2+}, which suggests class II PI3Ks bind lipids in a Ca^{2+} independent manner.

Class III are similar to class II in that they control the production of PI(3)P from PI, but are more similar to class I in structure, as they remain as heterodimer of a catalytic(Vps34) and a regulatory (p150) subunit. Class III seems to be principally involved in the trafficking of protein and vesicles. There is however, evidence that they are able to contribute to the usefulness of several process important to immune cells, not least phagocytes.

Mechanism

PI3-kinases formed 3-phosphorylated phosphoinositide which are (PtIlns3P, PtdIns(3,4)P$_2$ PtdIns(3,5)P$_2$ and PtdIns(3,4,50P$_3$) function in a mechanism by which various group of signalling protein, containing a pleckstrin homology domains (PH) PX domain, FYVE domain and other phosphoinositide-binding domains, are recruited to a variety of cellular membranes. PI3-kinases are inhibited by the worttmanin drugs except class 11 PI3-kinase family shows decreased sensitivity to the drugs.

An extraordinary varied group of cellular functions have been linked to PI3-kinase including cell growth, motility, proliferation, differentiation and intracellular trafficking. Many of these functions associate to ability of class 1 PI 3-kinase to activate protein kinase B (PKB, *Akt*). The class 1A PI 3kinase p110α is mutated in many cancers cells. M any of these mutations cause the kinase more active .The PtdIns (3,4,5)P$_3$ phosphatase

PTEN which atagonised PI 3-kinase signalling is absent from many tumors. The p110δ and p110γ isoform regulate different aspect of immune responses. PI 3-kinase plays a key role in insulin signaling pathway. Hence there is great interest in the role of PI 3-kinase signaling in diabetes mellitus. PKB activation require PI3-kinase signalling pathway in order to be translocated to the plasma membrane. At $P1P_3$, PKB is then phosphorylated by other kinases called phosphoinositide dependent kinase1 and 2 (PDK1) (PDK2), and is thereby activated.

The "PI3-k/*Akt*" signaling pathway has been shown to be needed for an extremely various selection of cellular activities - most notably cellular proliferation and survival.

1.9.1.4 Protein kinases PDK-1 PDK-2

PDK1 and PDK2 are distinct kinases in insulin signalling pathway. PDK1 is predominantly localized in the cytosol, whereas PDK2 is associated with both the plasma membrane (PM) and low density microsomes (LDM). Further characterization of the PM fraction using high salt extraction and differential centrifugation revealed that PDK2 activity co-sediments with a subpopulation of the PM fraction that is enriched in proteins associated with the actin cytoskeleton, including proteins present in focal adhesions but that this fraction is devoid of PDK1 activity (Richard *et al*.;2000). PDK2 is responsible for phosphorylation of *Akt* at Ser-473. The protein kinase responsible for phosphorylating *Akt* on Thr-308 is the recently identified phosphoinositide-dependent PDK1 (Alessi *et al*.; 1997).

1.9.1.5 Protein kinase B (PKB) / *Akt*

Akt is a serine/threonine kinase, has emerged as an essential enzyme in signal transduction pathways implicated in cell proliferation, apoptosis, angiogenesis, and diabetes. In mammals, three isoforms of *Akt* (*Akt* 1, 2, 3) are reported to show a high degree of sequence homology, but vary slightly in the localization of their regulatory phosphorylation sites. *Ak*t1 is the main isoform in most tissues, whereas the highest expression of *Akt*2 is observed in the insulin-responsive

tissues (Garofalo *et al*.; 2003) and *Akt*1 is abundant in brain tissue (Hemmings *et al*.; 2004). *Akt*2 is an important signaling molecule in the Insulin signaling pathway. It is required for insulin to stimulate glucose transport. The role of *Akt*3 is less clear, though it appears to be predominantly expressed in brain. It has been reported that mice lacking *Akt*3 have small brains (Hemmings *et al.;* 2004).

The activation of PKB by PI3K involves the recruitment of PKB to the plasma membrane through the interaction of PtdIns(3,4,5)P_3 with the PH domain, and phosphorylation by the phosphoinositide dependent kinase 1 (PDK1), which is also recruited to the membrane by the PH domain. PDK1 phosphorylates PKB at Thr308 in the catalytic domain (Mora *et al* 2004). PtdIns (3,4,5)P_3 is thought to promote the phosphorylation of PKB by PDK1 by causing a conformational change of PKB which exposes Thr308 and by bringing constitutively active PDK1 to the proximity of PKB (Alessi *et al* 1997 and 1998; Stokoe *et al* 1997; Anderson *et al* 1998). PKB is additionally phosphorylated at Ser473 in the C-terminal hydrophobic region ('the PDK2 site'). The identity of the kinase(s) that phosphorylates Ser478 is the subject of intense investigation (Dong and Liu 2005). Several candidate kinases, including mTOR/rictor complex, have been reported (Dong and Liu 2005). The Thr308 phosphorylation is essential for PKB activity. The role of Ser473 is less clear but it may stabilise the active configuration and/or it may assist the phosphorylation of Thr308 by PDK1 (Dong and Liu 2005). However, Thr308 phosphorylation has been seen in the absence of Ser473 (Morisco *et al* 2005).

In neonatal and adult rat cardiac myocytes, isoproterenol or β_2 receptor stimulation has been shown to increase PKB activity (as measured by GSK3α phosphorylation) and/or phosphorylation of Ser478 (Morisco *et al* 2000; Zhu *et al* 2001). It has been reported that phenylephrine does not affect Ser478 phosphorylation of PKB in adult rat cardiac myocytes, suggesting PKB may not be downstream of the α_1 receptor (Wang *et al* 2001). However, the lack of PKB phosphorylation under conditions that increase PI3K activity has been reported by several other studies (Till *et al*

2000; Zou *et al* 2002 and 2003) and it may be due to compartmentation of PI3K signalling. The activation of PKB by the β adrenergic receptor is sensitive to inhibitors of PI3K (Morisco *et al* 2000; Zhu *et al* 2001). However, several observations suggest that PKB may also be activated by PI3K-independent pathways. It has been shown in 293 cells that cAMP-raising agents activate PKB by a PKA-dependent but PI3K-independent pathway (Fillippa *et al* 1999). The activation of PKB by cAMP-raising agents was associated with increased phosphorylation of Thr308 but it was seen in the absence of Ser473 phosphorylation (Fillippa *et al* 1999). This is in contrast to the activation of PKB by insulin, which required phosphorylation of both Thr308 and Ser473 (Fillippa *et al* 1999). It appears that the increase in Thr308 phosphorylation by cAMP-raising treatments is not due to direct phosphorylation of PKB by PKA (Fillippa *et al* 1999). The possibility that PKB is also activated by the PKA pathway in cardiac myocytes is supported by the recent study by Morisco *et al* (Morisco *et al* 2005). These investigators showed that isoproterenol caused phosphorylation of PKB at Thr308 and Ser473 with different time-courses; the phosphorylation of Thr308 could be detected within 1 minute of stimulation and it returned to the basal level after 60 minutes, whereas the phosphorylation of Ser 473 increased after 10 minutes and was sustained for 2 hours (Morisco *et al* 2005). They showed that the early phosphorylation of Thr308 (10 minutes), but not the later phosphorylation of Ser473 (60 minutes), was insensitive to wortmannin (Morisco *et al* 2005). The phosphorylation of Thr308 was blocked by H89, nifidepine, or KN93, suggesting the possibility that PKA regulates PKB phosphorylation through activation of the L-type Ca^{2+} channel and CAMKII (Morisco *et al* 2005). How Ca^{2+}/CAMKII might be linked to PKB is not clear.
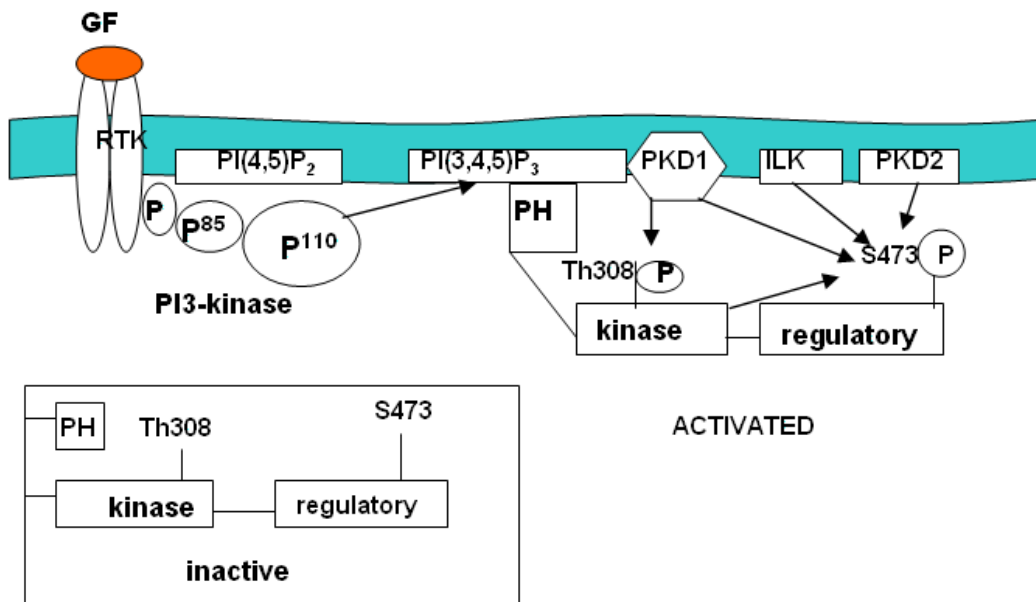
Figure 1.6: **Mechanism of activation of PKB/Akt**. The activation of PKB requires the phosphorylation of its both site Thr308 or Ser 473. Following growth factor (GF) activation of receptor tyrosine kinases (RTKs, or other cell surface receptors, not shown), PI-3K is recruited to the receptor and activated resulting in the production of PIP3. The translocation of PKB/Akt to the membrane where it is phosphorylated on Th308 within the catalytic domain by PDK-1 and on S473 within the regulatory domain by defined mechanism, possibly involving (a) autophosphorylation, (b) PDK-1, (c) integrin-linked kinase(ILK), or (d) an unidentified PDK-2. PKB/Akt is then released from the membrane and translocates to other subcellular compartments.

1.9.2 Catecholamines

Circulating catecholamines, adrenaline (A) and noradrenaline (NA), originate from two sources. A is released by the adrenal medulla upon activation of preganglionic sympathetic nerves innervating this tissue. This release activation occurs during times of stress (e.g., exercise, heart failure, hemorrhage, emotional stress or excitement, pain). Adrenergic agents increase the force and rate of heart contraction as well as decreasing the rate of heart relaxation.

During adrenergic stimulation of the heart, fatty acid, glucose oxidation and glycolysis are stimulated but glucose use is preferentially increased over fatty acid oxidation. In perfused working rat heart adrenergic stimulation increased ATP production by 50% and this was related with an increase in glycolysis, glucose oxidation and moderate 10% fatty acid oxidation (Collins *et al*.; 1994). Adrenaline increased glucose oxidation through an increase in Ca^{2+} and activation of PDH. The increase in glycolytic rate in response to adrenaline could be due to stimulation of PFK-2 via PKA phosphorylation.

NA is also released by the adrenal medulla (about 20% of its total catecholamine release is NA). The primary source of circulating NA is spillover from sympathetic nerves innervating blood vessels. Normally, most of the NA released by sympathetic nerves is taken back up by the nerves (some is also taken up by extra-neuronal tissues) where it is metabolized. A small amount of nonadrenaline, however, diffuses into the blood and circulates throughout the body. At times of high sympathetic nerve activation, the amount of NA entering the blood increases dramatically. Catecholamine content increases substantially in heart during cardiac infarction (Rundqvist *et al.;* 1997).

1.9.4 Adipokines

Adipokines (hormones secreted by adipocytes) either stimulate or inhibit AMPK activity. For example, adiponectin has been shown to stimulate AMPK activation, whereas resistin inhibits it.

1.9.4.1 Adiponectin

Adiponectin is a recently described adipokine that has been recognized as a key regulator of insulin sensitivity and tissue inflammation. It is produced by adipose tissue (white and brown) and circulates in the blood at very high concentrations. It has direct actions in the liver, skeletal muscle and the vasculature, with prominent roles in improving hepatic insulin sensitivity, increasing fuel oxidation [via up-regulation of AMPK activity] and decreasing vascular inflammation (Yamauchi *et al.;* 2002). Adiponectin increased AMPK α Thr172 phosphorylation in HL-1 atrial cardiac myocyte cells (Pineiro *et al*.; 2005). It exists in the circulation as varying molecular weight forms, produced by multimerization. Recent data indicate that the high-molecular weight (HMW) complexes undertake the predominant action in the liver. In contrast to other adipokines, adiponectin secretion and circulating levels are inversely proportional to body fat content. The levels are further reduced in subjects with diabetes and coronary artery disease. Adiponectin antagonizes many effects of tumour necrosis factor-alpha (TNF-alpha) and this, in turn, suppresses adiponectin production. Furthermore, adiponectin secretion from the adipocytes is enhanced by thiazolidinediones (which also act to antagonize the TNF-alpha effects). Two adiponectin receptors, termed AdipoR1 and AdipoR2, have been identified and these are ubiquitously expressed. AdipoR1 is most highly expressed in skeletal muscle and has a prominent action to activate AMPK, and hence promote lipid oxidation. AdipoR2 is most highly expressed in the liver, where it enhances insulin sensitivity and reduces steatosis via the activation of AMPK and increased peroxisome-proliferator-activated receptor alpha ligand activity. T-cadherin, which is expressed in the endothelium and smooth muscle, has been identified as an adiponectin-binding protein with a preference for HMW adiponectin multimers. Given the low

levels of adiponectin in subjects with the metabolic syndrome, and the beneficial effect of the adipokine in animal studies, there is exciting potential for adiponectin replacement therapy in insulin resistance and related disorders.

1. 9.4.2 Leptin

An adipocyte-derived hormone leptin has been shown to have different effects on AMPK in different tissues. Leptin activates α2 but not α1 AMPK in skeletal muscle (Minokoshi *et al.;* 2002). However, leptin inhibits AMPK in the hypothalamus (Andersson *et al.;* 2004; Minokoshi *et al.;* 2004), while it seems to have no effect on AMPK in the perfused heart (Atkinson *et al.;* 2002). The *in vivo* administration of leptin has been shown to activate AMPK by two pathways; the direct effect of leptin on skeletal muscle itself and an indirect slower effect through the hypothalamic-sympathetic nervous system (Minokoshi *et al.;* 2002). The direct effect of leptin seems to be mediated by the AMP-dependent pathway, as an increase in AMP was observed immediately after leptin administration (Minokoshi *et al.;* 2002). However, the delayed indirect effect is likely to be mediated by the α_1 adrenergic receptor via an AMP/ATP independent mechanism, as the *in vivo* effect was sensitive to phentolamine and phenylephrine activated AMPK in isolated skeletal muscle (Minokoshi *et al.;* 2002).

1.9.4.3 Resistin

Increasing resistin levels through peripheral infusion or transgenic overexpression impairs insulin action (Pravenec *et al*.; 2003). Resistin is a cysteine-rich protein implicated in insulin resistance and inflammation (Mojiminiyi and Abdella, 2007). The ablation of the *retn* gene or reduction of resistin via antisense oligonucleotide treatment enhances insulin sensitivity, leading to a decrease in hepatic glucose production and an increase in glucose uptake by the muscle and adipose tissue (Banerjee *et al*.; 2004). Furthermore, other studies suggest that an increase in resistin after high-fat feeding is the primary cause of hepatic insulin resistance (Muse *et al.;* 2004) by inhibiting the activity of AMPK (Kahn *et al*.; 2005).

1.10 Pathological alterations in cardiac energy metabolism

1.10.1 Diabetes

Diabetes mellitus is the metabolic syndrome characterizes by the body's inability to regulate blood sugar and lipid level. It is divided into two types.

Type 1 is characterized by the loss of insulin production beta cells of the islets of Langerhans in the pancreas, leading to the deficiency of insulin.

Type 11 is characterized by abnormal glucose and lipid metabolism because of insulin resistance inadequately compensated for by increased insulin secretion.

In uncontrolled diabetes, despite decrease carnitine content, the heart shows an increased dependence on fatty acid oxidation which is able to supply over 90% of the ATP required of the tissue (Wall and Lopaschuk 1989). High rates of fatty acids oxidation are due to the high plasma concentration of fatty acids in diabetic subjects, as well as decreased rates of glucose uptake from the circulation and an increase in the Km for glucose phosphorylation (Morgan *et al.;* 1961). The enhanced rates of fatty acids oxidation observed in diabetic heart has been proposed to involve the regulation of CPT1 by malonyl-CoA. No change in the sensitivity of CPT I 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 *et al.;* 1996). This decreases 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 *et al.;* 2000)

Increased rate-s of β-oxidation is 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 perfuced diabetic hearts compared to controls (Saddik and Lopaschuk 1994)

Diabetic hearts also have large store of TAG compared to healthy tissue, and this is probably due to an increase fatty acyl-CoA content which results from increase fatty acid availability and the increased synthesis of CoASH observed in diabetic hearts (Lopaschuk *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 and Lopaschuk 1994)

1.10.2 Cardiac Hypertrophy

The development of hypertrophy by the heart is associated with congestive heart failure, increased risk of myocardial infraction 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 *et al.;* 1999) and NADH (EI Alaoui-Talibi *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 heart (Kalsi *et al.;* 1999)

1.10.2.1 Physiological Hypertrophy

Heart continues to develop for a restricted period to adapt to increased workload. In particular after birth, the left side of the heart undergoes hypertrophy, while the right side actually gets slightly smaller due to changes in blood pressure throughout the heart that happens soon after birth. The first few breaths after birth fill the lungs with air, decreasing afterload on the right side of the heart and increasing pulmonary vascular blood flow. This increases blood flow to the left

side of the heart, increasing preload. Mean arterial blood pressure rises throughout the infant's body and increases the mechanical after load on the heart. It is the increased workload on the heart, particularly on the left side, that leads to increased heart size. The changes in cardiac size after birth result from both hypertrophy and hyperplasia of cardiomyocytes but as cardiomyocytes terminally differentiate during the first few weeks of life; further increases in heart size throughout life are due principally to hypertrophy (Oparil *et al.*; 1994).

Cardiac hypertrophy also occurs following (typically long-term) exercise training. The heart increases in size and mass subsequent to resistance training such as weight lifting, although there is some discussion as to whether true hypertrophy occurs in this scenario. Normalizing the increased heart weight to the increased body weight of the athletes appears to greatly reduce the increased ratio typically observed in true hypertrophy (Oakley, 1992) Nevertheless, resistance athletes frequently demonstrate some degree of concentric hypertrophy (increased wall thickness with normal chamber dimensions). In the case of isotonic training (e.g., distance running), cardiac hypertrophy is much more noticeable and often presents as an eccentric hypertrophy (increased wall thickness with increased chamber dilation) (Oakley *et al*.; 1992).

1.10.2.2 Pathological Hypertrophy

Pathological forms of cardiac hypertrophy commonly occur following acute events, such as myocardial infarction, or associated chronic abuse such as hypertension. In these examples, hypertrophy is thought to be an attempt to relieve increased transmural wall stress in the heart by thickening the wall (Morisco *et al.*; 2003). A range of inherited genetic disorders also results in cardiac hypertrophy as either a principal or secondary endpoint. For example, hypertrophic cardiomyopathy is an inherited disease that can result from a wide variety of genetic lesions, including mutations in contractile proteins such as ß-myosin heavy chain that may directly result in cardiac hypertrophy (Arad *et al.*; 2002). Alternatively, hypertrophy may represent a secondary

response to a distal lesion, such as that which occurs in pheochromocytoma, a tumor that wildly releases epinephrine and norepinephrine to continually activate adrenergic pathways in the heart (Prichard *et al.*; 1994). Pathologic hypertrophy thus typically represents an attempt by the heart to alleviate a stress or a response to unsuitable modification of prohypertrophic signaling pathways.

A number of signaling pathways has been involved in the regulation of cardiac hypertrophy, although the exact mechanism that causes hypertrophy has not yet been resolved. It has been proved that during chronic pressure overload AMPK activity increases (Tian *et al*.; 2006). This suggests that AMPK activation may cause a maladaptive alteration in the hypertrophy process or might be the cause of cardiac hypertrophy. The AMPK pathway has been involved in the regulation of myocyte growth. This increase in AMPK activity in the hypertrophied heart also increases glucose uptake which accelerates glycolytic ATP production (Tian *et al*.; 2001). In some situations, AMPK activation would appear to be helpful to heart. By contrast to this AMPK activation can become an enemy to cardiac function. The role of AMPK in the hypertrophic process may be variable depending on the severity of hypertrophy.

1.10.3 Ischemia

Ischaemic heart disease, which is a common cardiovascular disease, cruelly endangers health and is a most important focus of medical studies (Lazzarino *et al.;* 1994; Pierce & Czubryt, 1995) that are carried out at the level of the whole body (Toyoda *et al*.; 2000), whole heart (Cerniway *et al*.; 2002), tissue (Nishioka *et al.;* 1984) and single cardiomyocyte (Louch *et al*.;2002). The use of varied ischaemia-mimetics and models of cardiac ischaemic injury are essential to research into this condition.

Cardiac ischemia is associated with an increase in AMP/ATP and activation of AMPK through the phosphorylation of Thr172 (Kudo *et al* 1995; Baron *et al.;* 2005). Sakamoto and co-workers studied the role of LKB1 in the activation of AMPK during ischemia using transgenic mice lacking cardiac expression of LKB1 (Sakamoto *et al.;* 2006). The deletion of LKB1 in the heart prevented the increase in the Thr172 phosphorylation of AMPK in response to ischemia, suggesting that LKB1 mediates the activation of AMPK during ischemia (Sakamoto *et al.;* 2006). However ischemia has been shown to have no effect on the catalytic activity of LKB1, as measured by *in vitro* phosphorylation of recombinant AMPK by LKB1 immunoprecipitated from the ischemic heart, or on the protein abundance of LKB1, although LKB1 purified from the heart is able to phosphorylate α312 (the truncated catalytic subunit which can be phosphorylated and activated in the absence of AMP), confirming the constitutive activity of LKB1 (Sakamoto *et al.;* 2006; Altarejos *et al.;* 2005). Thus, as suggested for other AMP/ATP raising conditions, the phosphorylation of Thr172 by LKB1 during ischemia is also likely to be due to the effect of AMP to inhibit the dephosphorylation of AMPK by protein phosphatase.

Recent studies suggest that in addition to LKB1, the activation of AMPK during ischemia also involves some other AMPKK which, unlike LKB1, is activated by ischemia. In the aforementioned study by Sakamoto and co-workers, whereas the Thr172 phosphorylation of α2

AMPK induced by ischemia was completely abolished, ischemia could still increase the Thr172 phosphorylation of α1 AMPK in LKB1-deficient hearts (Sakamoto *et al.;* 2006). This suggests that LKB1 is the major AMPKK mediating the α2 AMPK activation during ischemia but α1 AMPK is also activated by some other AMPKK, as well as by LKB1 (Sakamoto *et al.;* 2006). Other groups found that although LKB1 can be completely precipitated with 5% PEG 6000 and it is not present in the supernatant, AMPKK activity still remained in the supernatant and this AMPKK activity was increased by ischemia (Altarejos *et al.;* 2005). Moreover, Baron *et al* have shown that the total AMPKK activity present in a heart homogenate is increased by ischemia (Baron *et al.;* 2005). Given that LKB1 activity is not increased by ischemia, this result further supports the idea that the activation of AMPK during ischemia involves an AMPKK whose catalytic activity is activated by ischemia. Whether this AMPKK is a CAMKK or a yet unidentified AMPKK is unknown. The mechanism by which ischemia activates this AMPKK also remain to be determined. However, AMP does not appear to activate this AMPKK as the total cardiac AMPKK in the study by Baron *et al* was not directly activated by AMP *in vitro* (Baron *et al.;* 2005).

Recent studies have revealed that mild ischemia or hypoxia increases α-Thr172 phosphorylation and AMPK activity without affecting AMP/ATP or Cr/CrP (Altarejos *et al*.; 2005; Frederich *et al.;* 2005). This raises the possibility that during severe ischemia, AMPK is activated by two parallel pathways; the AMP pathway and a pathway which is independent of AMP but dependent on some factors that are generated or activated during mild ischemia or hypoxia. Despite the lack of changes in the high energy phosphates, Altarejos *et al* still observed the activation of the non-LKB1 AMPKK activity present in the 5% PEG 6000 supernatant by mild ischemia (Altarejos *et al.;* 2005). Thus, this non-LKB1 AMPKK may be responsible for the activation of AMPK during hypoxia/mild ischemia and for AMP-independent activation of AMPK during severe ischemia. However the involvement of LKB1 in the AMP-independent activation of AMPK during ischemia/hypoxia cannot be ruled out because it is conceivable that 'non-AMP factors' may

stimulate the phosphorylation of AMPK by LKB1 by modifying the interaction of AMPK with LKB1 or by modifying a property of AMPK such as the affinity of AMPK for AMP. Supporting the latter possibility, that hypoxia increases the sensitivity of AMPK to stimulation by AMP (Frederich *et al.;* 2005).

1.11 Aims of the Project

It is important to regulate the fuel consumed by the heart. During normal conditions, fatty acids are the most dominant fuel of the heart. High levels of fatty acids are detrimental to the ischemic myocardium and during reperfusion. Glucose is the principal fuel during numerous types of stress such as ischemia in which glycolysis is the only source of ATP for the heart. Regulation of fatty acid and glucose metabolism is essential under these conditions. Numerous studies have investigated the effects of AMPK in the regulation of energy metabolism in the heart during normal and pathological conditions. Recent studies have proved that AMPK can be regulated by hormones and nutrients.

The aim of this project was to investigate the effect of glucose on AMPK phosphorylation in cardiac myocytes. This effect has previously been investigated in skeletal muscle (Samar I *et al*.;2003), where it was found that increasing glucose concentration caused inactivation of AMPK and a decrease in AMPK phosphorylation at α-Thr172. The approach in the present study was to investigate this effect on cardiac myocytes and to initially determine whether the effect is due to glucose or its metabolites. Glucose has multiple functions, when it is transported into the cells. It is phosphorylated to generate glucose 6-phosphate, which can give rise to many metabolic products.

The objective of this study was to verify which pathway of glucose has the potential to inhibit AMPK activity and phosphorylation at α-Thr172 and then to establish the mechanism by which glucose affects AMPK activity and phosphorylation.

Alteration of glucose metabolism occurs in the heart in response to hormones and nutrients. An additional aim was to test the relationships between fructose 2,6-bisphosphate, glycogen concentration and the activity of PFK-2 and AMPK under the same conditions that were used in the previous experiments (cells incubated with different glucose concentrations, with and without).

Cardiac myocytes were used as the experimental system for this study because myocytes from the same heart can be incubated under multiple conditions.

Chapter -2- Materials and Methods

Materials and Methods

2.1 Commercial preparations

Commonly used reagents were obtained from Sigma-Aldrich or BDH (VWRI international). The following were supplied by:

Sigma-Aldrich: (-)-Epinephrine, BSA (essentially fatty acid free, fraction V), palmitic acid sodium salt, protein G-Sepharose, 2-deoxy-D-glucose, phenazine methosulfate, trans-dehydroandrosterone, 3-O-methyl-D-glucopyranose, glucose 6-phosphate disodium salt, D-fructose 2,6-bisphosphate, tetrasodium salt, D-ribulose 5-phosphate, 3-epimerase, fructose 6-phosphate kinase, β-nicotinamide adenine dinucleotide reduced disodium salt, lactic acid sodium salt, xylulose 5-phosphate, thiamine pyrophosphate, bicinchoninic acid protein assay kit, xylitol and Azaserine.

BDH : Purified water used for cardiac myocyte isolation.

BOC Ltd : O_2:CO_2 (95%:5%) cylinders.

Merial : Sodium Pentobarbitone.

Worthington Biochemical Corporation: Collagenase Type 2 , hyaluronidase.

Roche: DNaseI, NADH, disodium salt, $NADP^+$, disodium salt, Glucose 6-phosphate Dehydrogenase, Aldolase, Amyloglucosidase, GDH/Triosephosphate Isomerase, NAD^+ cryst lithiumsalt, Glyceraldehyde 3-phosphate Dehydrogenase, Pyruvate, Fructose 6-phosphate.

Fluka : D-xylulose, D-ribose 5-phosphate disodium salt, D-ribose.

American Radiolabeled Chemicals : D–glucose[1-^{14}C], D-glucose[2-^{14}C], D-glucose[6-^{14}C], D-glucose[3/4-^{14}C].

Wako Chemicals Inc: NEFA C Test kit.

Calbiochem (Merck Biosciences): AICAR, oligomycin, Okadaic Acid and Endothall.

Santa Cruz Biotechnology: Anti- α2 AMPK antibody (goat).

Cambridge Research Biochemical s: SAMS peptide.

N.E.N: [γ-^{33}P] ATP, [9,10-^{3}H] palmitate, [U-^{14}C] glucose.

Cell Signalling Technology : Anti-phospho-Thr172 AMPKα antibody (rabbit), anti-phospho-Ser485/491 AMPKα antibody (rabbit), anti-AMPKα antibody (rabbit), anti-phospho-Ser79 ACC1 antibody (rabbit), anti-ACC antibody (rabbit), anti-phospho-Thr308 PKB antibody (rabbit), anti-phospho-Ser473 PKB antibody (rabbit), anti-PKB antibody (rabbit), HRP-linked anti-rabbit secondary antibody.

Rockland : RDye^{TM}800-conjugated anti-rabbit secondary antibody.

Invitrogen: NuPAGE® 4-12% Bis-Tris gels, PVDF membranes. Buffers used for SDS-PAGE and Western blotting (NuPAGE® LDS Sample Buffer, Sample Reducing Agent, Antioxidant, MOPS SDS Running Buffer, and Transfer Buffer).

2.2 Laboratory preparations

2.2.1 Palmitate bound to albumin

Palmitate was bound to albumin by a modification of the method described by Evans and Muller (1963). Sodium palmitate was added to a solution containing 15% (w/v) of bovine serum albumin (BSA), and 0.9% (w/v) NaCl (250 mg of sodium palmitate was added per 50 ml of the solution). The mixture was sonicated in a sonicating water bath until an even suspension was obtained. The mixture was incubated at 50°C for 30 minutes, followed by incubation at 4°C overnight. Undissolved sodium palmitate was removed by filtration through Whatman No.1 filter paper at 4°C, followed by centrifugation at 26,000g for 30 minutes at 4°C in a Beckman Sorvall RC5-B centrifuge. The supernatant was collected and the pH was adjusted to 7.4 with NaOH. After determining the concentration of palmitate bound to albumin (**Section 2.11**), the preparation was aliquoted and stored at -20°C.

2.2.2 AMPK antibody bound to protein G

Protein G sepharose was washed and resuspended as a 50:50 slurry in a 50 mM Hepes buffer pH 7.4 containing 50 mM NaF, 5mM $Na_4P_2O_7$, 10% (v/v) glycerol, 1mM EDTA and 1mM dithiothreitol (DTT). 100 μl of α2 AMPK antiserum, or pre-immune sheep serum as a control, was added to 500 μl of slurry and mixed åC4for 2 hours. After binding, the slurries were washed twice in Hepes buffer as above and stored at 4˚C until used.

2.3 Animals

The animals used in these experiments were male Sprague-Dawley rats bred in the animal colony at University College London. The rats selected for experimentation had a 250-300g body weight (6-8 weeks old). They were maintained at 20-22˚C and had constant access to drinking water and Rat and Mouse Breeding Diet (Special Diet Services, Witham, Essex, UK). The diet contains 21% protein, 4% fat and 39% starches and sugars (by weight). The light/dark cycle was 13hr/11hr, with light from 06:00 to 19:00hr.

2.4 Heart perfusion

Rats were anaesthetised by an intraperitoneal injection of sodium pentobarbitone (300mg/Kg). The hearts were removed and placed in ice-cold Krebs-Henseleit Bicarbonate Buffer (KHB) that had been freshly-prepared (25 mM $NaHCO_3$, 18.5 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, pH7.4, 3 mM $CaCl_2$ and 5 mM glucose and pre-gassed with O_2/CO_2 (95:5). This was followed by a retrograde perfusion through the aorta, as previously described (Mowbray and Ottaway 1973), using KHB containing 2% (w/v) BSA, 1.3mM $CaCl_2$. The concentration of glucose used in the perfusion buffer varied (0, 5, 10 or 25 mM) depending on the experimental conditions. The perfusion buffer was constantly gassed with $O_2:CO_2$ (95 %:5 %) and maintained at 37°C. The perfusion pressure (preload) was 70 ± 5 cm H_2O and the perfusion rate was approximately 15 ml/min. All hearts were initially perfused for 5 minutes in non-recirculation mode to remove blood and endogenous hormones. Perfusion was then continued for a further 60 minutes, with recirculation of the perfusate. At the end of the perfusion, the hearts were removed from the cannula and freeze-clamped using tongs pre-cooled to the temperature of liquid N_2. The frozen hearts were powdered under liquid N_2 and stored in liquid N_2.

2.5 Rat cardiac ventriceular myocyte isolation

Myocytes were isolated using a slight modification of the method described by Fuller *et al* (1990*)*. The buffers used in the isolation procedure were Buffer A consisting of KHB + 10 mM glucose + 1.3 mM $CaCl_2$, Buffer B consisting of KHB + 10 mM glucose + 0.1 mM EGTA, Buffer C consisting of KHB + 10 mM glucose + 0 to 5 μM added $CaCl_2$, Wash Buffer consisting of KHB + 10 mM glucose + 0.2 mM $CaCl_2$ + 2 % BSA + 0.5 mg/ml DNase I, and resuspension Buffer consisting of KHB + 1.3 mM $CaCl_2$ + 2 % BSA + 5 mM glucose. All buffers were prepared with purified H_2O, with minimal contamination with Ca^{2+}. The buffers (except for ice-cold Buffer A) were maintained at 37°C and constantly gassed with $O_2:CO_2$ (95%:5%). The rats were anaesthetised and their hearts were removed and put in ice-cold buffer A, followed by a brief washout perfusion at 37°C with Buffer A, as described above. After perfusion with

recirculating buffer A for 10 minutes, the perfusate was switched to Buffer B, and Ca^{2+} was washed from the heart. Perfusion with Buffer B was continued for a further 5 minutes, followed by washing with Buffer C to remove EGTA. The hearts were then perfused with Buffer C, containing 0.1 % (w/v) BSA, 1 mg/ml collagenase (type 2), and 0.4 mg/ml hyaluronidase for 20 to 30 minutes until they became soft. The exact amount of Ca^{2+} added to Buffer C (<5 μM) depended on the batch of digestive enzymes and the level of Ca^{2+} contamination. The myocytes were gently dispersed in perfusion Buffer C containing 0.1 % BSA, 1 mg/ml collagenase, 0.4 mg/ml hyaluronidase, and 0.5 mg/ml DNase I by shaking in a water bath set at 37 ℃ (~150 oscillations/min) for 5 minutes with gasing. DNase I was included to minimise the loss of myocytes due to the clumping of the cells by DNA released from dead cells. The digested heart was filtered through nylon mesh and the cells were allowed to settle under gravity for 5 minutes at 37℃. The supernatant, which contained non-settled dead cells, was removed and the cell pellet was resuspended in Wash Buffer. The myocytes were again allowed to settle under gravity for 3 minutes. The supernatant was removed and the cells were resuspended in fresh Wash Buffer. The Ca^{2+} concentration was then gradually increased every 2 minutes, from 0.4, 0.6, 0.8, 1.0 to 1.3 mM, while the cells were shaken gently at 37℃ with gassing. Finally, the myocytes were allowed to settle under gravity for 5 minutes and the cell pellet was resuspended in Resuspension Buffer (cells: buffer 1:10 (v/v)) as a Cell Stock.

2.6 Measurement of cardiac myocyte viability

Myocyte preparations containing more than 70% viable cells were used for metabolic studies. The viability was assessed using a haemocytometer and Trypan Blue (0.4%) which stains non-viable cells. Viable cells which excluded Trypan Blue were of a typical rod shape with visible cross striations. Non-viable cells did not exclude Trypan Blue and were rounded with no distinct ultrastructure. Each preparation was assessed for viability before use, and cells which had less than <70 % viability were discarded.

2 .7 Preparation of myocytes for individual experiments.

The myocytes were incubated in 18 ml plastic scintillation vials under different conditions, as described in each individual experiment. At the end of the incubation period, the cells were centrifuged at 500xg for 1 minute, then washed twice with free BSA Buffer and stored under liquid N_2 for the measurement of metabolite or enzyme activity. For the flux studies, the cells were treated as described in **section 2.8.** The diagram below illustrates the usages of myocyte for various experiments: (**Figure 2.1**)
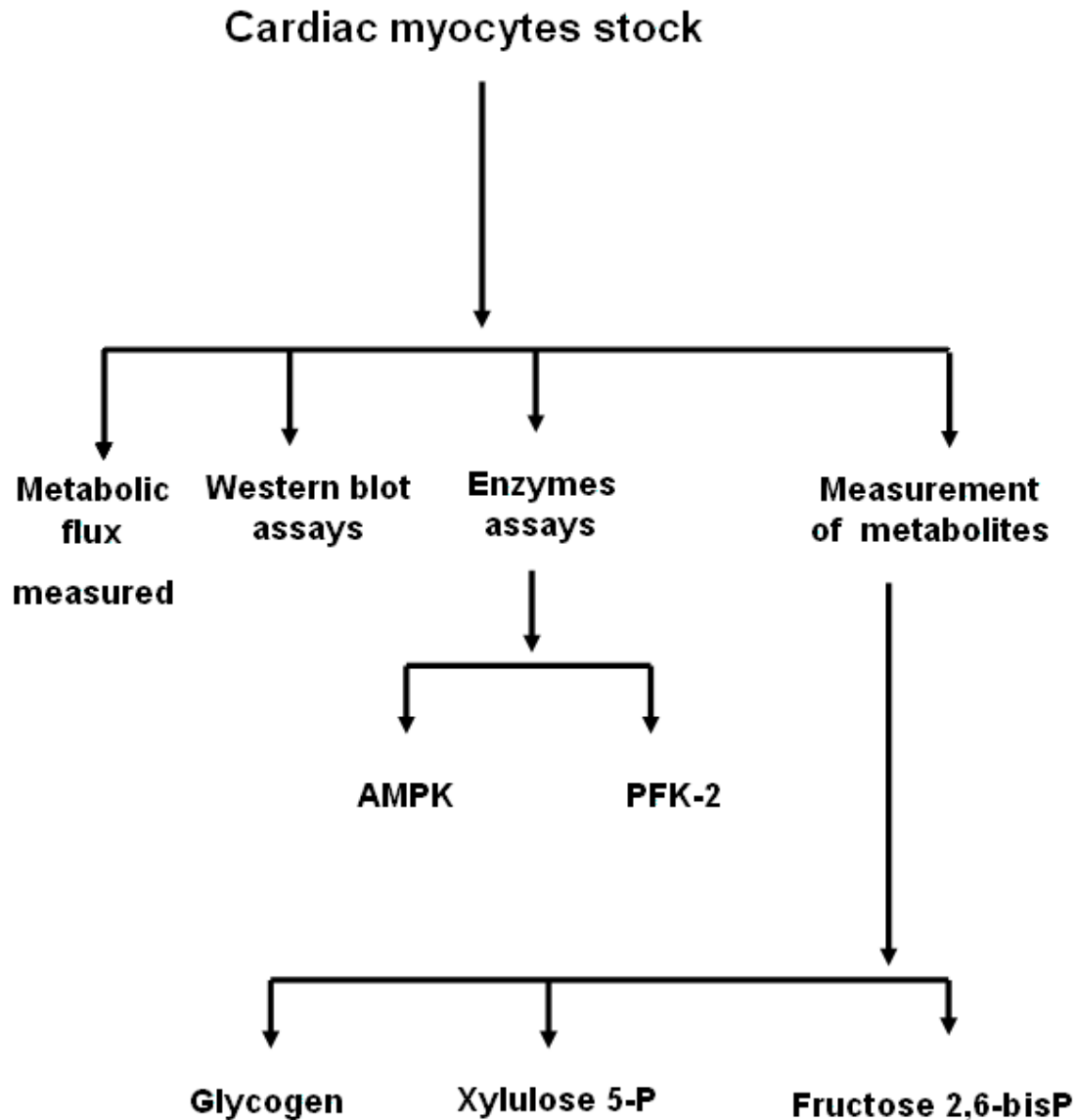
22.8 Measurement of glucose oxidation by myocytes

The incubation buffer (KHB) which contained 5 mM [U-^{14}C] glucose (approximately 20 μCi/mmol) was gassed with (O$_2$:CO$_2$) before use. Myocytes were incubated at 37ºC, with gentle shaking in a total volume of 2.5 ml (0.5 ml Cell Stock + 2 ml buffer) in sealed 50 ml siliconised glass metabolic flasks fitted with centre wells. At the end of the indicated incubation period, 0.2 ml of 60% perchloric acid (PCA) and 0.5 ml of 1M benzethonium hydroxide was injected through the stopper to the outer compartment (containing myocytes) and the centre well, respectively. Flasks were incubated at 37°C, with shaking for a further 60 minutes in order to collect [^{14}C]CO$_2$ (method of CO$_2$ trapping). The contents of the centre wells were transferred to 18 ml plastic scintillation vials. The centre wells were then rinsed twice with 0.5 ml methanol, which was then also transferred to the scintillation vials and 10 ml of Ecoscint A was added. The radioactivity was measured by liquid scintillation counting. Similar experiments were conducted using different labelled glucoses (1-^{14}C, 6-^{14}C, 2-^{14}C, and 3/4-^{14}C).

In order to eliminate background counts due to fluorescence, the vials were stood in the dark overnight before counting.

2.9 Determination of metabolite contents

2.9.1 Glycogen

Glycogen was extracted by boiling cell pellets **(section 2.7)** in 5 ml glass tubes containing 1 ml of 0.5 mM K0H for 20-30 minutes until these were judged to be completely digested by observing an even suspension. The mixture was then vortexed and cooled on ice. Glycogen was precipitated from the alkaline digest by adding 2 ml of cold 95% (w/v) ethanol. The mixture was vortexed and placed on ice for 30 minutes and then centrifuged for a further 30 minute at 5000g. The supernatant was discarded and the tubes inverted on tissue to drain for several minutes. The pellets were used for the determination of glycogen.

Glycogen hydrolysis

The above pellets were placed in 5 ml stoppered glass tubes and dissolved in 100 µl of water, followed by the addition of 50 µl of 43 mM $KHCO_3$ and 1.0 ml of glucoamylase solution. The mixture was incubated at 4℃ for 2h in a shaking water bath, and then 0.5 ml of 210 mM perchloric acid was added, mixed well and centrifuged at 5000g for 10 minutes. The supernatants were neutralised with solid $KHCO_3$, centrifuged again. The supernatant was used for glucose determination.

Glucose determination

Glucose released from the hydrolysis of myocyte glycogen was measured by the method of (Devos P *et al.;* 1983)).

Principle of the assay :

(a) $(glycogen)_n$ + n-1 H_2O $\longrightarrow$ n glucose

(b) Glucose + ATP $\xrightarrow{\text{HK}}$ Glucose 6-P + ADP

(C) Glucose 6-P + $NADP^+$ $\xrightarrow{\text{G6P-DH}}$ 6P-gluconolactone + NADPH + H^+
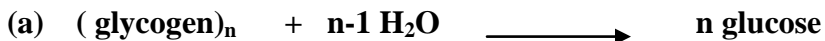
Assays were carried out in duplicate in a final volume of 1.50 ml in a 3 ml plastic cuvette containing 0.5 ml sample, 1.0 ml of assay mixture that contained 50 mM Tris- HCl buffer (pH 8.0) 0.5 mM ATP, 0.2 mM $NADP^+$ and 0.02% (w/v) BSA. The blank cuvette contained 0.5 ml of water and 1.0 ml of assay mixture. The blank and sample cuvettes were placed in the spectrophotometer. 10 µg of glucose phosphate dehydrogenase was added to both cuvettes (to remove any glucose 6-phosphate that might have been present in the cell extracts). The absorbance was read at 340 nm. When a steady reading had been obtained, the reaction was initiated by adding 10 µg hexokinase and the increase in the absorbance was read after 5-10 minutes until a constant absorbance value had been obtained. The glycogen content was

expressed in mM glucosyl units (the molar extinction coefficient of NADH at 340 nm is 6.22x 10^3 L. mol^{-1}.cm$^{-1)}$.

Radioactive method for measuring glycogen

Tissues were weighed, and then transferred into 15 ml vials containing 5 ml KHB Buffer and 5 µCi D-[U-^{14}C] glucose. The vials were transferred to the shaking water bath adjusted to 37℃ and gased continuously for an hour. The tissue was removed and frozen in liquid nitrogen. The tissue was then ground to a powder under liquid nitrogen. The powder was homogenized in a 10 ml tube containing 2 ml of 3 M KOH, then heated at 70℃ for 30 minutes. After cooling the homogenate to room temperature, 4.7 ml of 100% ethanol was added, followed by storage at -20 ℃ overnight. Glycogen was precipitated by centrifugation at 5000 g for 10 minutes. The precipitate was suspended in 2m of 90% ethanol, vortexed, and then recentrifuged. This last step was repeated once more. The precipitate was suspended in 0.2 ml water followed by 4 ml scintillation fluid, and the radioactivity was measured.

2.9.2 D-Fructose 2,6-Bisphosphate

Preparation of cardiac myocyte extracts for assay of fructose 2,6-bisphosphate:

5- 10 mg samples of frozen myocytes were placed in 2 ml plastic tubes and homogenised in 1.0 ml of 0.1 M NaOH. The pH of the resulting mixture should be above 11.0 to denature protein and stabilize fructose 2,6-bisphosphate. The homogenates were heated for 5 min at 80℃, then cooled and centrifuged at 6000g for 5 minutes. The resulting supernatants were neutralized at 0℃ with 1.0 M ice-cold acetic acid in the presence of 20 mM Hepes pH 7.0. This results in the precipitation of protein. Fructose 2,6-bisphosphate was assayed in the last supernatants. When the sample contained endogenous inhibitors, it was necessary to purify the extract by adding 1-2 ml to a column of Dowex AG1x8 (4 x 40 mm Cl$^-$ form). The column was washed with 1 ml water and 3 ml of 0.15 M NaCl, then F-2,6-P$_2$ was eluted in 2 ml of 0.3 M NaCl.

Fructose 2,6- bisphosphate was measured spectrophotometrically by the method of (Van *et al.*; 1980).

Principle of the assay:

$$\text{F 2,6-P}_2$$

$$F\ 6\text{-}P\ +PP_i \quad \underset{+}{PPi\ PFK} \quad F\ 1,6\text{-}P2\ +\ Pi$$

$$F\ 1,6\text{-}P_2 \quad \xrightarrow{ALD} \quad GAP\ +DHAP$$

$$GAP \quad \xrightarrow{TIM} \quad DHAP$$

$$DHAP\ +\ NADH\ +H^+ \quad \xrightarrow{GDH} \quad glycerol\text{-}3\text{-}P\ +\ NAD^+$$
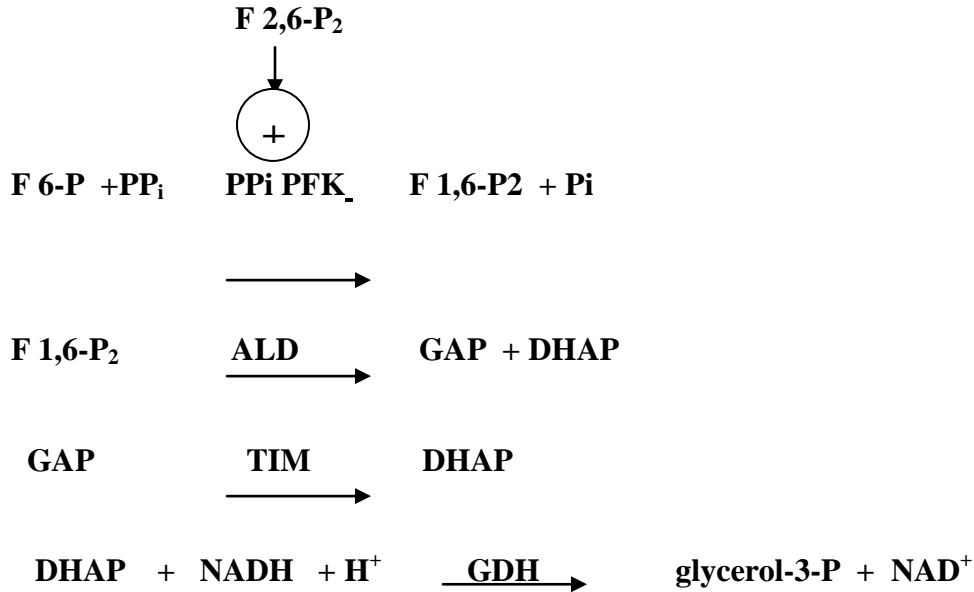
Assays were carried out in duplicate at $25°C$ in a final volume of 1 ml containing 50 mM Tris Buffer (pH 8.0), 2 mM magnesium acetate, 1mM F-6-P, 0.15 mM NADH, 6 ug PPiPFK, 40ug (Glycerophosphate dehydrogenase/ triosephosphate isomerase) (GDH/ TIM), F2,6-P2 < 10 nmol and 0.5mM $PP_{i.}$ The mixture was vortexed well and incubated for 5 minutes at $25°C$. The ini tial absorbance was read at 340 nm using a Unicam SP8-100 spectrophotometer against a blank containing water in place of the sample. The reaction was initiated by the addition of 0.05 ml of a 10 mM pyrophosphate solution. The decrease in absorbance was measured at approx 10 minutes after the start and used to calculate the Fructose 2,6-bisphosphate concentration. The sample should not contain more than 1 picomol of Fru-2,6-P2. A calibration curve was established with 20, 50, 100 µl from a 10 nM F-2-6P2 solution, corresponding to 0.2, 0.5 and 1 picomol, respectively.

For the standards, the changes in absorbance were plotted versus the corresponding concentration of fructose 2, 6-bisphosphate, pmol/ml (**Figure 2.2**)
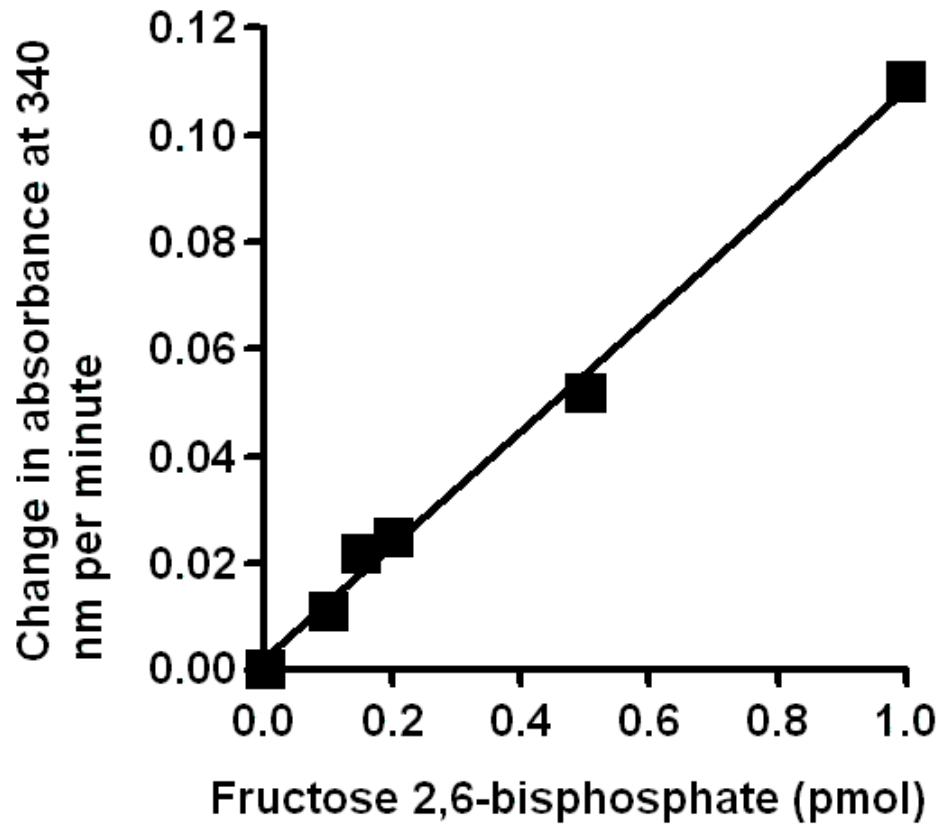
Figure 2.2: Fructose 2,6-bisphosphate standard curve The standard curve showing the average absorbance for the concentration range between 0.1 to 1.0 pmol of fructose 2,6-bisphosphate.

2.9.3 Xylulose 5-Phosphate (Xu5P)

Preparation of tissue extracts for xylulose 5-phosphate measurement

10 mg samples of frozen myocytes were placed in 5 ml glass tubes containing 1 ml of (10% w/v) trichoroacetic acid, then vortexed well, followed by centrifugation for 10 minutes at 10,000g. The supernatants were transferred to fresh tubes and neutralized by addition of 1 M $NaHCO_3$. The precipitates were removed by centrifugation and the supernatants were used directly for Xylulose 5-Phosphate determination.

Xylulose 5- phosphate was assayed spectrophotemetricaly by method of (Harvey D.J and Horning M.G 1976).

Principle of the assay:

(a)   **Xu5-P + R5-P** $\xrightarrow[\textbf{TPP,Mg}^{2+}]{\textbf{TK}}$ **G3-P + Sedoheptulose 7-P**

(b)   **G3-P** $\xrightarrow{\textbf{TIM}}$ **DHAP**

(c)   **DHAP + NADH + H$^+$** $\xrightarrow{\textbf{GDH}}$ **α- Glycerol Phosphate** **+ NAD$^+$**
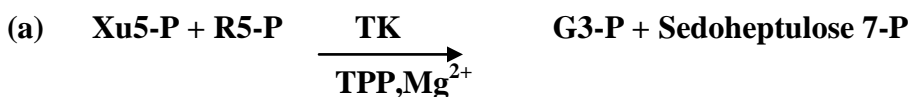
The assay was carried out at 25°C in a final volume of 1 ml containing 250 mM glycylglycine buffer (pH 7.7), 50 mM ribose 5-phosphate 30 mM thiamine pyrophosphate, 4.3 mM NADH, 0.3 M $MgCl_2$, 4 µl from Glycerophosphate dehydrogenase/ triosephosphate isomerase (10 mg/ml) was freshly prepared, and 0.6 ml of cell extract. The mixture was allowed to equilibrate at 25°C. The reaction was initiated by the addition of 10 µl of 5.0 unit/ml transketolase and the change in absorbance at 340nm was recorded. Readings were taken against a reagent blank where the samples were replaced with water.

2.9.4 Measurement of adenine nucleotide contents

Adenine nucleotides were measured by ion-pair reverse phase HPLC, as described by Sellevold *et al* (1986), using a 150 X 4.6 mm Nucleosil C18 column with a particle size of 5 μm and a pore size of 100Å. The mobile phase consisted of 215 mM KH_2PO_4, 2.3 mM tetrabutylammonium hydrogen sulphate (TBAHS), and 3.5 % (v/v) acetonitrile. The pH was adjusted to 6.25, with 10 M KOH. The mobile phase was filtered through a 0.22 μm Millipore filter and degassed before use. 200 μl of ice-cold 0.21 M perchloric acid was added to the frozen myocytes and the mixture was vortexed. Following centrifugation at 13,000 g for 5 minutes at 4 ºC, perchlorate ion in 150 μl of the supernatant was precipitated with 30-40 μl of 1M KOH, which was added in sufficient amounts to precipitate most of the perchlorate in the extract whilst maintaining an acidic pH (6.25). The precise amount was determined by the titration of an extract and the usual amount was 20-40 μl. Potassium perchlorate precipitates were removed by centrifuging at 13,000 g for 2 minutes at 4 ºC, and 45μl of the mobile phase was added to 50 μl of the supernatant. The samples diluted with the mobile phase were filtered through a 0.22 μM filter and 50 μl was injected onto the column which was pre-equilibriated with the mobile phase. Adenine nucleotides and creatine compounds were eluted isocratically at ambient temperature. The flow rate was 1.0ml/min and each run lasted for 20 minutes. The elution of the creatine compounds and the adenine nucleotides was detected by changes in the absorbance at 206 nm using a Waters tunable absorbance detector. The elution profile was stored and processed using Millennium 2010 software. The peaks for ATP, ADP, and AMP were identified by comparing the retention times with standards (**Figure 2.3).** In order to ensure that the standards had similar amounts of residual perchlorate as the samples, the former were prepared in 0.21 PCA, which was partially neutralised with the same amount of 1 M KOH as used in the preparation of the latter, and then diluted ten times with the mobile phase before injection onto the column. The amounts of adenine nucleotides and creatine compounds were determined by the comparison of the peak

areas and peak heights respectively with those of the standards. In order to establish whether this method is sufficiently sensitive accurately to measure the small amount of adenine nucleotides present in the myocyte samples, the standards were prepared in the range of concentrations similar to those seen in the myocyte samples (**Figure2.3)**
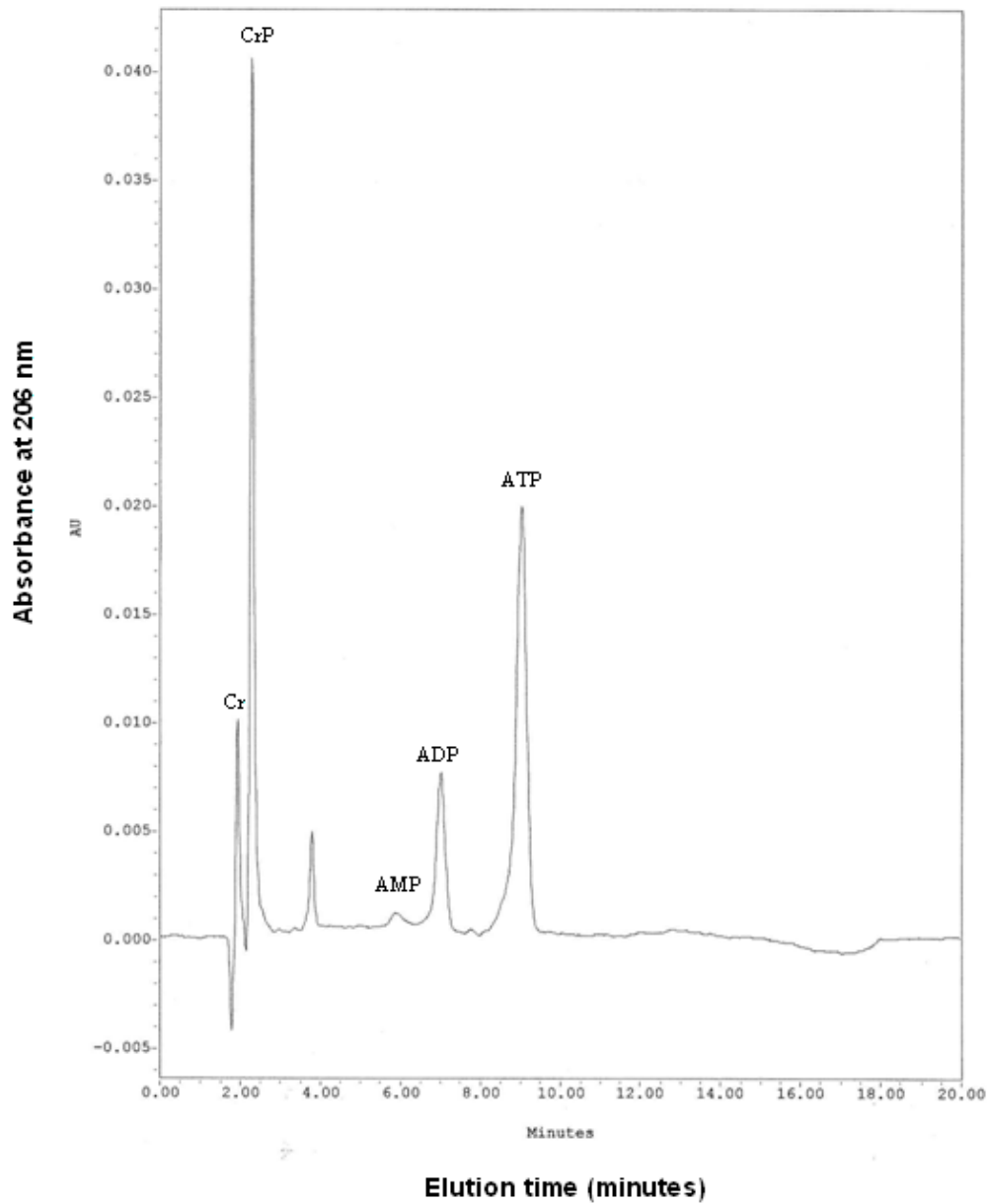
Figure 2.3: ATP:ADP:AMP :Cr : CrP.

A chromatogram of standard mixture of ATP,ADP,AMP, creatine, and phosphocreatine
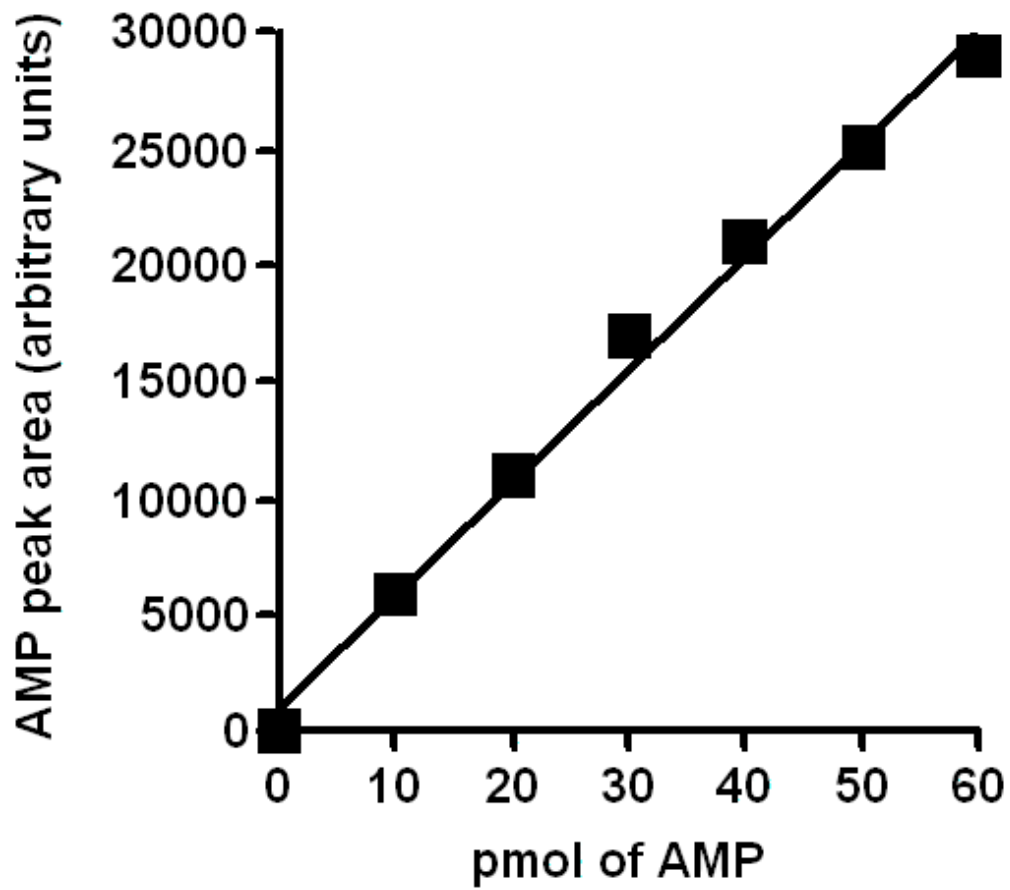
Figure 2.4: AMP standard curve. AMP standard solution was analysed by HPLC as described in **section 2.9.4** with the indicated concentrations. This result is from a single set of measurements.

2.10 Measurement of protein concentration

Protein concentration was determined by a bicinchoninic acid (BCA) method. This method is based on the concentration-dependent reduction of alkaline Cu(II) to Cu(I) by proteins, and the chromogenic reaction of BCA with Cu(I). 10 μl of the samples was incubated at $37^{\circ}C$ for 30 minutes with 200 μl of BCA, copper (II) sulphate pentahydrate 4% solution (50:1, v/v) in a 96 well microtitre plate. After incubation, the absorbance at 550 nm was determined using a Dynatech MR7000 spectrophotometer. BSA solutions (0.2-1mg/ml) were used as standards
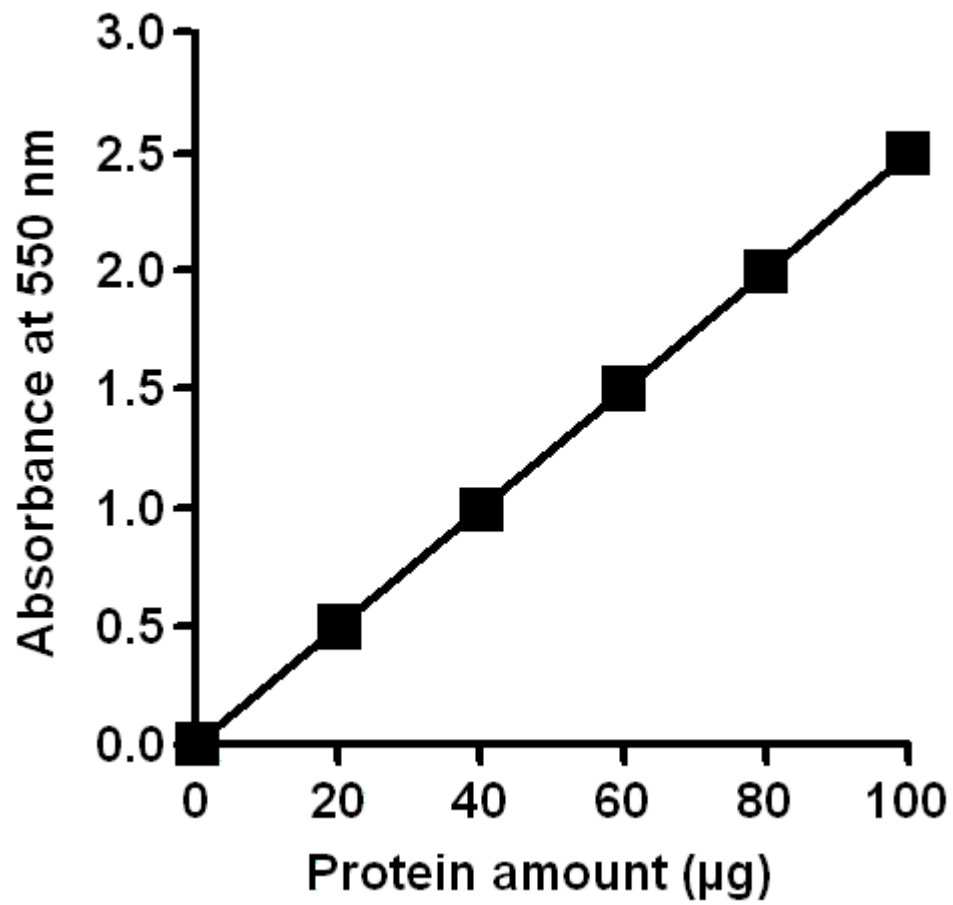
Figure 2.5: **Protein standard curve.** The standard curve shows net absorbance at 550 nm versus the indicated protein concentrations for a single test assay.

2.11 Determination of non-esterified fatty acid concentration

Non-esterified fatty acid concentration was assayed using a Wako NEFA C test kit. This assay is based on the oxidation of fatty acyl-CoA by acyl-CoA oxidase (ACOD) to 2,3-trans-enoyl-CoA. This reaction releases H_2O_2, which, in the presence of peroxidase and 4-aminoantipyrine, allows the conversion of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) to a coloured compound. 10 μl of the samples was incubated in a 96 well microtitre plate at 37°C for 10 minutes with 67 μl of Reagent A (50 mM phosphate buffer [pH 6.9] containing 3 mM $MgCl_2$, 0.3 U/ml acyl-Coenzyme A synthetase, 3 U/ml ascorbate oxidase, 3 mg/ml ATP, 0.7 mg/ml coenzyme A, and 0.3 mg/ml 4-aminoantipyrine). 133 μl of Reagent B (1.2 mM MEHA, 6.6 U/ml ACOD and 7.5 U/ml peroxidase) was then added and the samples were incubated for a further 10 minutes. The absorbance at 550 nm was determined with a Dynatech MR7000 spectrophotometer. Oleate solutions (0.2-1mg/ml) were used as standards.

2.12 Enzyme Assays

2.12.1 AMPK activity assay

Frozen myocytes were sonicated (3 x 7 seconds) in 0.3 ml of ice-cold sonication buffer containing 50 mM Tris/HCl(pH 7.5), 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM $Na_4P_2O_7$, 10% glycerol (v/v), 1% Triton X-100 (v/v), 1 mM DTT, 1mM phenylmethanesulfonyl fluoride (PMSF), 1 mM benzamidine, and 4 μg/ml soybean trypsin inhibitor. The cell lysate was centrifuged at 13,000g for 10 minutes at 4°C and 60 μl of the supernatant was incubated at 4°C for 2 hours with either anti-AMPK antibody or pre-immune serum prebound to Protein G-Sepharose (**Section 2.2.2**). The volume of antibody/Protein G Sepharose used for immunoprecipitation was 20 μl for α2 AMPK. This volume was chosen because it was found to optimize immunoprecipitate AMPK from the 13,000xg supernatant (**Figure 2. 9).** After binding, the immunoprecipitates were collected by centrifugation at 5,200g for 1 minute at 4°C, and washed once with 200 μl of sonication buffer and twice with AMPK assay buffer (40mM Hepes

(pH7.0), 80 mM NaCl, 0.8 mM EDTA, 8% glycerol (v/v), and 0.8 mM DTT) by centrifuging as above. The immunoprecipitates were then incubated at 37°C with gentle shaking in a final volume of 50μl of AMPK assay buffer, which additionally contained 200 μM SAMS peptide (HMRSAMSGLHLVKRR), 200 μM [γ-^{33}P] ATP (100-500 dpm/pmol), 5 mM MgCl$_2$, and 200 μM AMP. The reaction was started by the addition of [γ-^{33}P] ATP. The assay which measures incorporation of ^{33}P to SAMS peptide (Davies *et al* 1989) is based on the sequence surrounding the AMPK phosphorylation site (Ser79) of ACC1, with a mutation of Ser77 to alanine to prevent phosphorylation by PKA and the addition of two arginines to allow recovery of SAMS peptide by ion exchange after the reaction. The reaction was stopped after 30 minutes by spotting the reaction mixture onto squares of P81 Whatman phosphocellulose ion exchange paper which were then immersed in 1% (v/v) orthophosphoric acid. The SAMS peptide phosphorylated with ^{33}P was separated from [γ-^{33}P] ATP by washing the papers twice in 1% orthophosphoric acid followed by two washes in distilled H$_2$O (10 minutes each). The papers were air-dried and transferred to scintillation vials, and 10 ml of Ecoscint A was added before radioactivity was determined by liquid scintillation counting. Because α-2 AMPK antibodies was raised in goats, activity immunoprecipitated with sheep/goat pre-immune serum prebound to Protein G-Sepharose was used as a blank to account for the non-selective binding of AMPK with serum proteins and/or Protein G Sepharose, and was subtracted from the activity immunoprecipitated with anti-AMPK antibodies.AMPK showed a good linear correlation with amount of 13,000g protein in the range of the amount typically used (**Figure 2.10).** AMPK activity also increased linearly during the assay time (30 minutes) (**Figure 2. 11).**

For heart samples, frozen, powdered heart was weighed and homogenised on ice using an Ultra Turrax tissue disintegrator in 1 ml per 100 mg of tissue homogenisation buffer containing 50 mM Tris/HCl (pH 7.8), 0.25 M mannitol, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5mM Na$_4$P$_2$O$_7$, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, and 4 μg/ml soybean trypsin inhibitor. 13,000 g

supernatant was obtained and AMPK was immunoprecipitated and assayed as above, except for the fact that the immunoprecipitate was washed with Homogenisation Buffer instead of Sonication Buffer.
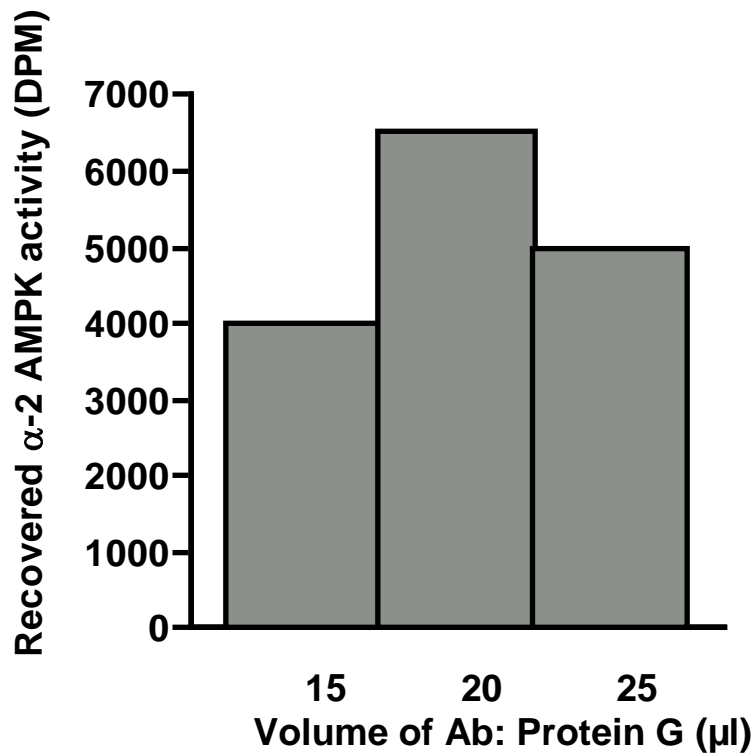
Figure 2.6: **Effect of altering antibody concentration on the Immunopreciptate of α2 AMPK activity.** α-2AMPK was immunoprecipitated with the indicated volumes of Ab: Protein G. The assay was carried out in the presence of 200µ AMP. Values are as dpm per minute for one myocyte preparation.
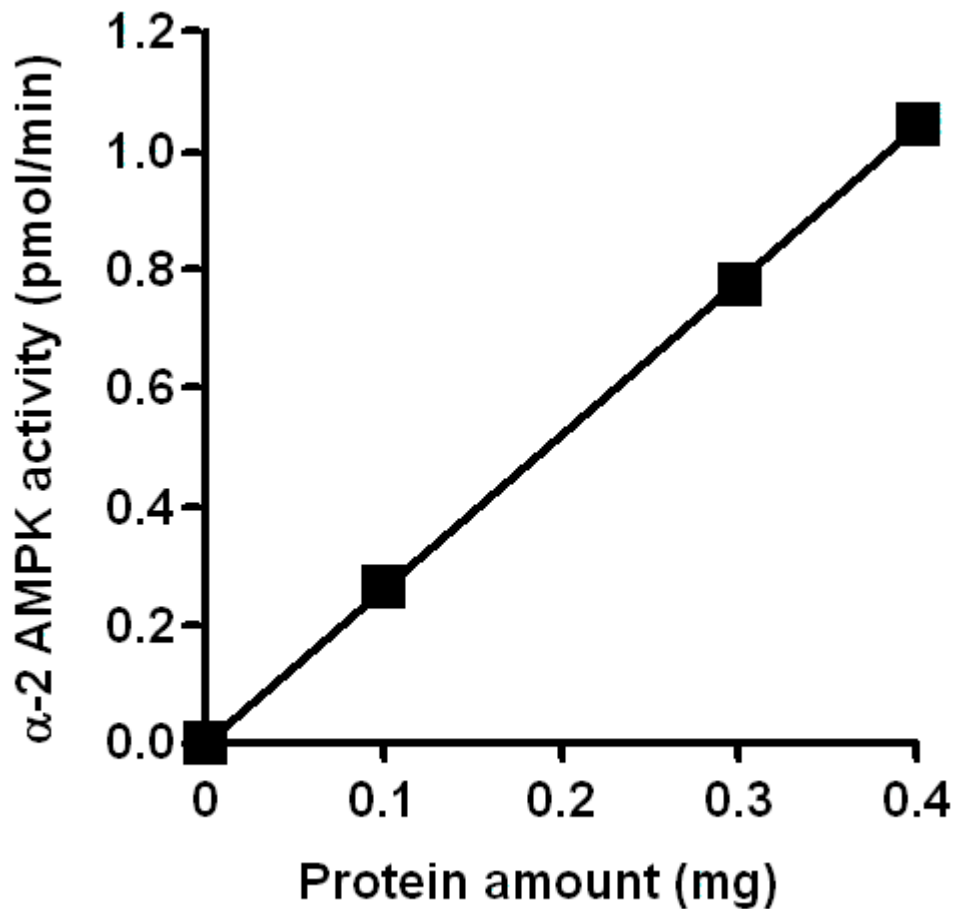
Figure 2.7: **the relationship between AMPK activity and the amount of protein in the 13,000g supernatant**. Cardiac myocytes were incubated for 1hr with KHB buffer. Cells were then lysed and centrifuged at 13000xg. The supernatant was then collected and different protein concentrations were prepared from the same myocyte preparation. α2 AMPK was immunoprecipitated from the supernatants and AMPK activity was assayed in the presence of 200 µM AMP as described in section **(2.12.1)**
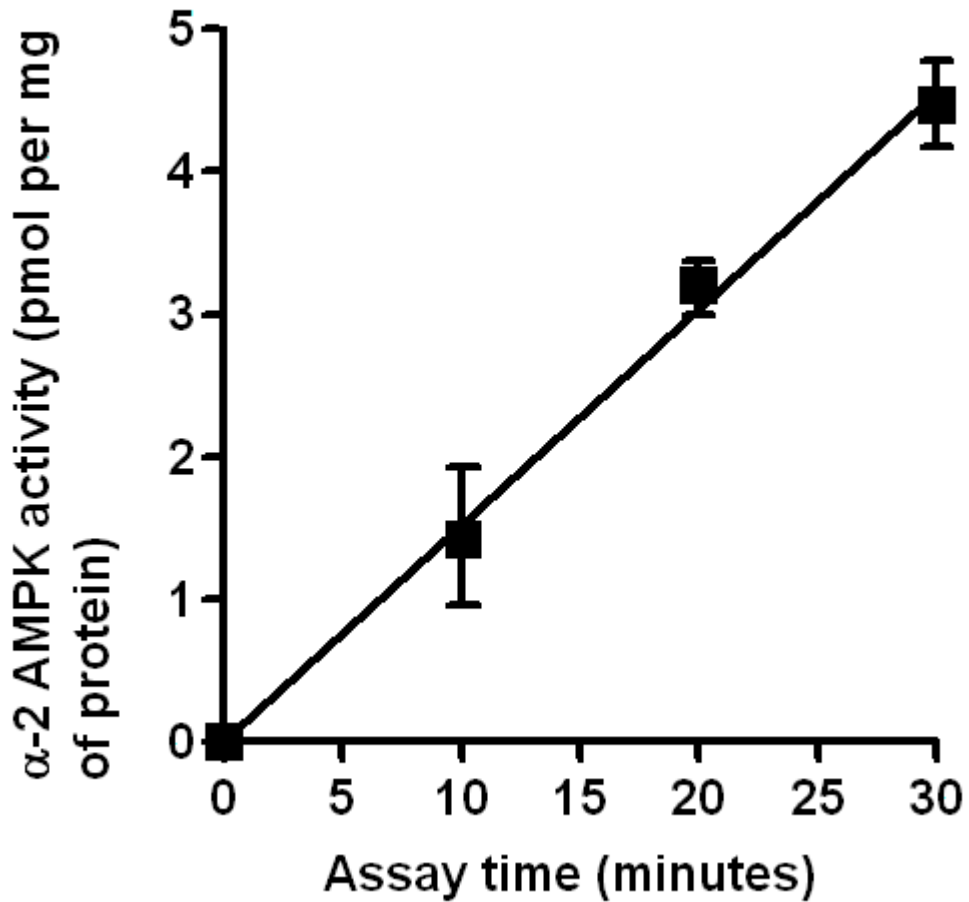
Figure 2.8: Time course of AMPK activity. α2 AMPK was immunoprecipitated from cardiac myocytes 13,000xg supernatant and assayed for AMPK activity in the presence of 200 μM AMP. Values are mean ± SEM of 5 independent measurements of myocyte preparation.

2.12.2 Phosphofructo-2-kinase

For the determination of PFK-2 activity, cells suspensions were thawed in 50 µl of buffer containing 20 mM-Pi, 10 mM- EDTA and 100 mM-KCl [pH 7]. The cell extracts were centrifuged at 10,000g. Each assay was conducted in a 1 ml final volume containing 0.95 ml of (50 mM Hepes Buffer [pH 7.1], 1 mM ATP 100 mM KCl, 5 Mm $MgCl_2$, 5 mM Fru-6-P, 3.5 mM Glc-6-P, 0.1 mM AMP) and 50 µl of cell extract. The reaction was terminated after 10 minute incubation at 30°C, by the addition of 100 µl of 0.1 M NaOH, then cooled and centrifuged. The supernatant was taken for the measurement of Fructose 2,6-bisphosphate as described in **Section 2.9.2**. The activity was calculated from an extinction coefficient of 6.22x 10^3 $L.mol^{-1}.cm^{-1}$ for NADH and as expressed as nmol/min/mg dry weight of cells. For the time course, the reaction was terminated at 2, 4, 6 and 8 minutes (**Figure 2.12).**
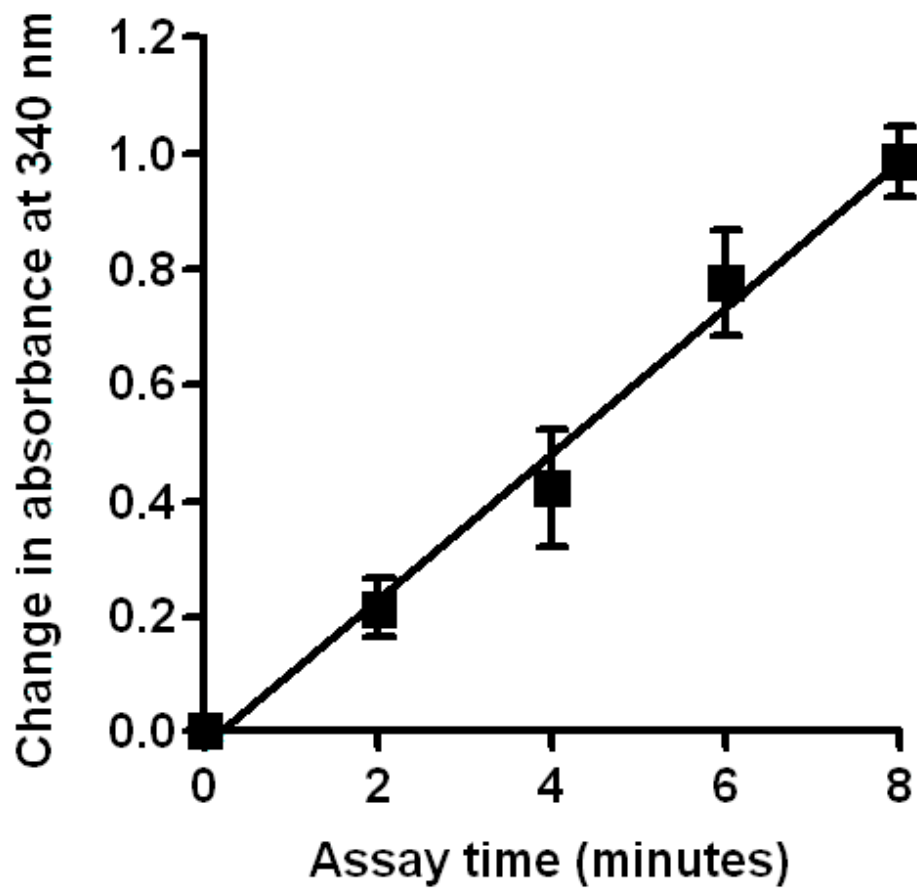
Figure 2. 9: **PFK2 activity time course.** Cardiac myocytes were incubated for
1 hour at 37 °C in the presence of 5 mM glucose and 2 % BSA. Cell extracts were
assayed for PFK2 activity for the indicated times as described in **section (2. .9 .2).**
The results are expressed as mean ± SEM for 8 cell preparations.

2.13 Western blotting

Myocytes were lysed as desecribed in **Section 2.12.1** and the proteins in 15-25 μl of the 13,000 g supernatant were separated by sodium dodecylsulphate polyacrylamide gel electrophoreses (SDS-PAGE) under denaturing and reducing conditions on 4-12% Bis-Tris gels, and transferred to polyvinyldene difluoride (PVDF) membranes. Phospho-AMPK α-Thr172, Phospho-AMPK α-Ser485/491, Phospho-PKB Thr308, Phospho-PKB Ser473, Phospho-ACC Ser79/227, total AMPKα, total PKB, and total ACC were detected by chemiluminescence (Fujifilm LAS-1000 Image Reader) with horse redish peroxidase (HRP-) conjugated secondary antibody and chemiluminescent substrate (luminol). For these measurements, primary and secondary antibodies were used at a 1/1000 dilution in Tris Buffered Saline (TBS) (20 mM Tris/HCl pH7.6, 137 mM NaCl) containing 5% (w/v) BSA and 0.1 % (v/v) Tween-20. The secondary antibody used for the detection of Ser485/491 was diluted in the same buffer which additionally contained 0.01 % SDS. 5% (w/v) milk powder in TBS + 0.1% Tween-20 was used to block membranes except for those probed with anti-AMPK Ser485/491, which were blocked with 5% BSA in TBS + 0.1% Tween-20. Band intensities were quantified with Fujifilm Multi Gauge software. After the measurements of Phospho-AMPK, ACC, or PKB, the membranes were stripped by incubating with 62.5 mM Tris/HCl buffer, pH 6.7, containing 0.8% (v/v) ß-mercaptoethanol and 2% (w/v) SDS at 50°C for 30 minutes, and then reprobed with total AMPK, ACC, or PKB antibodies.

2.14 Liquid Scintillation counting

The radioactivity of ^{14}C, ^{3}H, and ^{33}P was measured using a Perkin Elmer Tri-Carb 2900TR Liquid Scintillation Analyzer, which was equipped with software which made correction for chemical quenching and provided data as disintegrations per minute (dpm).

2.15 Statistical methods and representation of results

All values are given as means ± S.E.M. for the numbers of independent myocyte preparations shown in the legends to figures and tables.

Statistical significance was determined by Student's t tests. For comparisons of data from perfused hearts and for myocyte adenine nucleotide contents an unpaired test was used. All other comparisons of data from myocytes were done by means of a paired test. Throughout $P≤0.05$ was taken as the threshold of statistical significance.

In some instances measurements of AMPK activity are given as absolute values but in many experiments AMPK activities were normalized by expression as percentages ± S.E.M. of the paired activity that was measured with 5 mM glucose alone (basal activity). Error bars shown on figure symbols for these 5 mM glucose basal values indicate the S.E.M. of the absolute values expressed as a percentage of the mean of the absolute values. For all other conditions individual values were first expressed as percentages of the paired basal value followed by calculation of means ± S.E.M. This form of data representation was particularly used for measurements of myocyte AMPK activity in order to facilitate easy comparison of effects in different experiments because AMPK activity measurements were made throughout the whole time frame of the project and showed some temporal 'drift' in their absolute values. This was possibly due to variations in animals or antibody reagents. Measurements of other quantities were usually performed within a much smaller time frame and did not suffer from this 'drift'. From 106 independent preparations of myocytes incubated with 5 mM glucose alone the α2 AMPK activity assayed in the presence of 200 µM AMP was 2.06 ± 0.17 pmol per min per mg of 13,000g supernatant protein. Although the mean absolute AMPK activities could be rather disparate between experiments performed at different times the ranges of AMPK activities within a given experiment generally were not large. For example the experiments recording the highest and lowest mean α-2 AMPK activities with 5

mM glucose gave values of 4.44 ± 0.40 (n = 8) and 0.57 ± 0.04 (n = 9) pmol per min per mg of 13,000g supernatant protein respectively. For comparison mean α-2 AMPK activities in rat hearts perfused for 1 hour with 5 mM glucose ranged from 1.8 to 4.2 pmol per min per mg of 13,000g supernatant protein (Figure 5 and Table 1 respectively of Clark *et al.,* 2004).

Chapter - 3- Results

Section: 1

3.1 Isolation and viability of Cardiac Myocytes

In previous years, several methods have been developed for the isolation of cardiac myocytes. Many of the most successful methods involve perfusion and digestive enzymes, such as collagenase. The inability of isolated myocytes to maintain structural and metabolic integrity in the presence of Ca^{2+} (Farmer *et al.*, 1977; Vahouny *et al.*, 1979) has been a major problem in the isolation of viable myocytes from heart tissue. This problem has been overcome by perfusing the heart tissue in Ca^{2+} free buffer in the presence of EGTA, and digestion with a combination of collagenase and hyaluronidase. Myocytes are usually isolated under conditions of low Ca^{2+} which assists their release from intercellular connections and the extracellular matrix (ECM). It has been reported that the gradual reintroduction of Ca^{2+} to the myocytes via a series of washes can reduce the loss of viability caused by the Ca^{2+} paradox. Cardiac myocytes isolated by the method described in **section 2.5. Figure3** shows the cardiac myocytes visualized by light microscopy**. Figure 3.2.** Myocyte viability was determined as described in **section 2.6.** In this chapter, incubation conditions are described for each individual experiment where appropriate and stated in figure legends.

To assess the metabolic performance of isolated myocytes, glucose oxidation by myocytes was determined. Linearity of the rates of glucose oxidation for at least 1 hour indicated that the cells are metabolically active and there is no significant loss of viability during this period (**Figure 3.3).** The value for the rate of glucose oxidation was roughly in the range of that reported by other investigators (Montini *et al.;* 1981), an oxidation rate with 5 mM glucose of approximately 1.4 nmol/mg protein. Burns and Reddy reported an oxidation rate with 5 mM glucose approximately 0.7 nmol/ min/mg protein. The rate of glucose oxidation (5 mM) obtained in the present study also was 0.7 nmol/ mg protein.

Figure 3 .1: Isolated cardiac myocytes as visualized by light microscopy. The ratio of rod-shaped cells compared with rounded cells gives an indication of cell viability
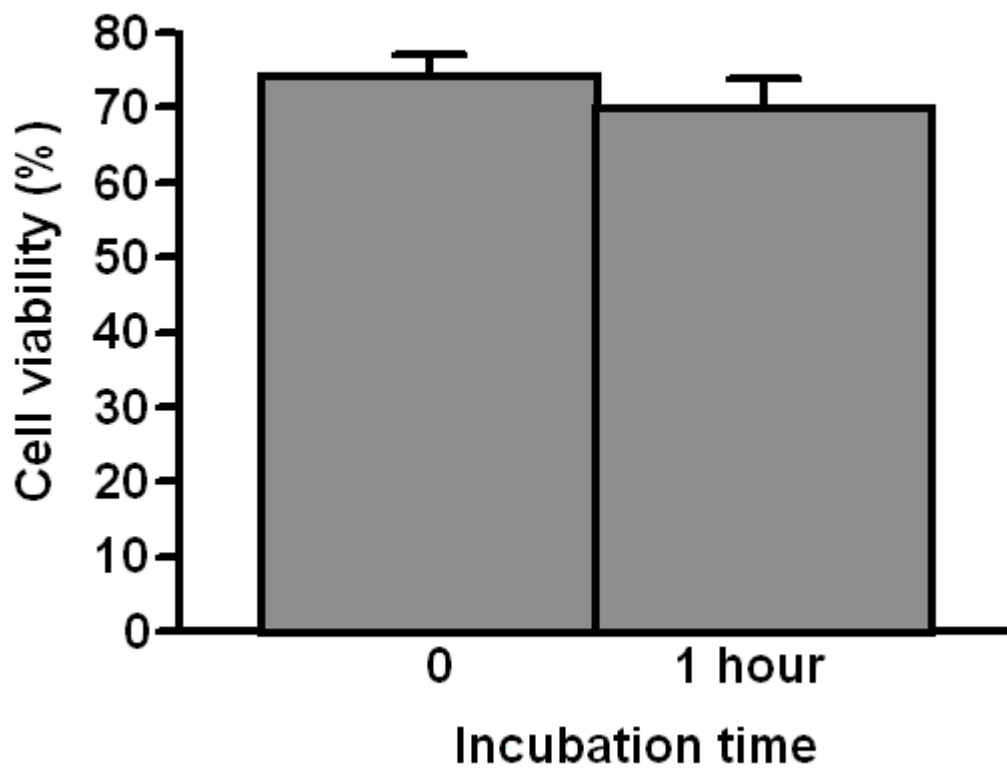
Figure 3.2: Assessment of the viability of cardiac myocytes. Cardiac myocytes were incubated with 5 mM glucose and 2% BSA. The cell viability was assessed as described in **section 2.6.** The values are the mean ± SEM of 3 independent preparations.
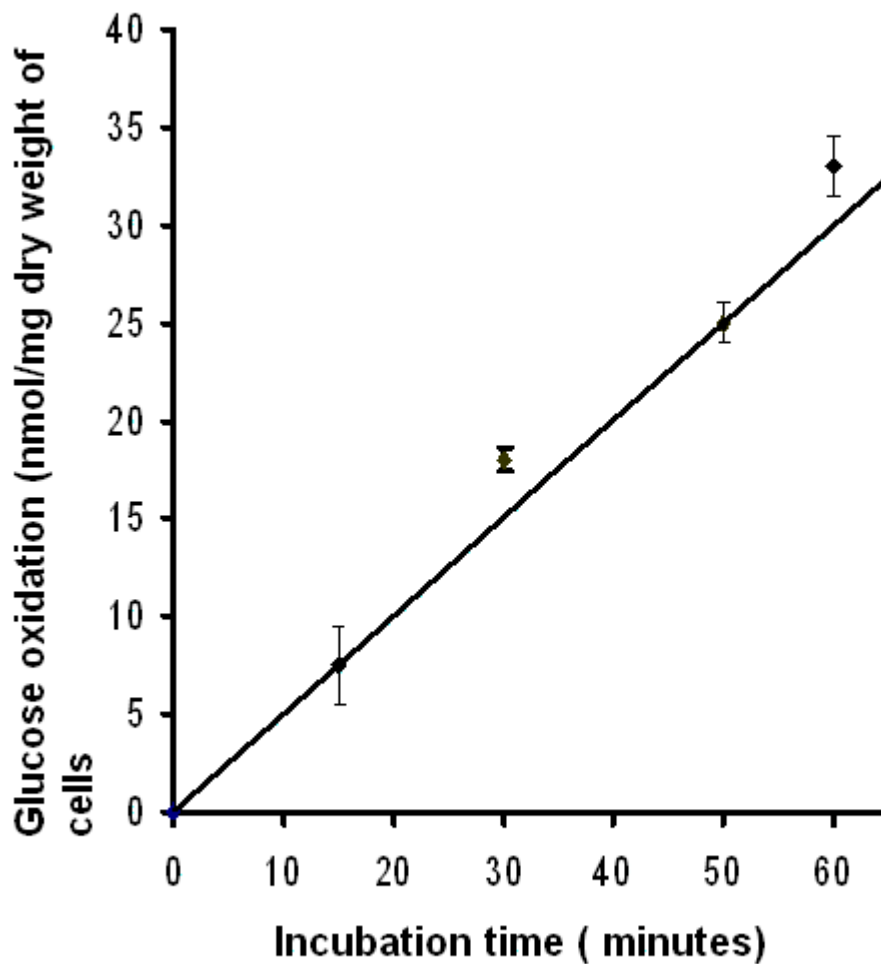
Figure 3.3: Glucose oxidation by cardiac myocytes. Cardiac myocytes were incubated for the indicated times in the presence of 5 mM [U-^{14}C]glucose. Glucose oxidation was estimated from $^{14}CO_2$ production as described in **section 2.8.** The values are means ± SEM of 4 independent myocyte preparations.

3.1.1 Conclusions

An isolation procedure which produced a good yield of uncontaminated, viable cardiac myocytes is essential for any biochemical investigation of myocardium at cellular level. The isolation procedure used in this study has been used successfully by previous members of the   Saggerson laboratory for a number of years and has been shown to produce cardiac myocytes which are viable both morphologically and biochemically.

The linearity of glucose oxidation with time indicated that the cells viability was sustained. The combination of a good myocyte isolation procedure with reliable metabolites assays, allows reliable measurements of the metabolites to be made in cardiac myocytes.

Section: 2

3.2.1 Myocyte AMPK activity is decreased by increasing glucose concentration.

A previous study by Itani *et al.* in 2003 found that, when rat skeletal muscles were incubated with increasing glucose concentrations AMPK activity and phosphorylation at α-Thr172 decreased in response to the increasing glucose concentration with no observed difference in the total AMPK protein levels. This decrease in AMPK activity was linked with a decrease in glucose transport. In addition, there were no changes in the concentration of adenine nucleotides or creatine-P during the incubation period (Itani *et al.;* 2003). It has been reported in the yeast Saccharomyces cerevisiae Snf1 is regulated by glucose depletion, which triggers its phosphorylation at Thr210 and concomitant increase in activity (Portillo *et al.*; 2005). Inactivation of AMPK by glucose has also been previously described in pancreatic β-cells and could be a pivotal regulatory event in glucose-induced lipogenesis (Salt *et al.;* 1998). In order to investigate this further, and examine whether or not this effect could be reproduced in cardiac myocyte, it was first necessary to isolate cells with good viability. Cell viability was determined as described in **section 2.6.** Second in this experiment, freshly isolated cardiac myocytes were subjected to several washings with washing buffer but no glucose in the last wash, in order to create a zero glucose baseline. Cardiac myocytes were then incubated for 1 hour in media containing 0, 2, 5, or 25 mM glucose. Since it was impossible to remove all glucose from the incubation medium and myocytes prior to incubation, the 0 mM glucose incubation contained a small unknown amount of glucose. Finally after incubation, the cells were lysed and the AMPK was immunoprecipiated using anti-AMPK α2 polyclonal antibody. Immunoprecipitated AMPK was used in an *in vitro* ^{33}P AMPK kinase assay using SAMS peptide as a substrate, as described in **section 2.12.1.** Both α-1 and α-2 AMPK subunit are expressed by rat heart with the α-2 complex being responsible for 70-80% of kinase activity (Cheung *et al.;* 2000). Measurement of AMPK activity was therefore confined to study of α-2 AMPK.

Figure 3.4 shows the effect of varying glucose concentration on AMPK α-2 activity. Glucose at 2, 5, 25 significantly decreased   α-2 AMPK activity compared to the control (zero glucose) by 70%, 52% and 18% respectively   when measured in the presence of 200 µM AMP (Suter *et al.;* 2006). All AMPK assay were performed in the presence of 200 µM AMP. Since the half-stimulation effect of AMP is less than 2 µM (Suter *et al..;* 2006) , this concentration of AMP will be essential to give maximal stimulation of the enzyme. Assay of AMPK with AMP present has the following advantages (i) it might be possible to assay the enzyme in the total absence of AMP because the possibility of breakdown of ATP to AMP either on storage or during the assay;(ii) the induction of AMP makes the assay more specific for AMPK and (iii) it increase the activity and thereby increases the   precision of the assay.

The effect of glucose on AMPK need to be examined further to see whether glucose promotes an allosteric effect or dephosphorylation of such a site or other additional mechanisms might exist that leads to an inactivation of AMPK by glucose.

3.2.2 The effect of glucose on α-2Thr172 phosphorylation

AMPK is phosphorylated and activated by upstream kinases. Thr-172 has been identified as the major site within the α catalytic subunits to be phosphorylated by these kinases (Hawley *et al.;* 1996).   To determine whether phosphorylation of this residue was decreased in response to increased glucose concentration, immunoblotting experiments were carried out. Samples of 13,00g supernatants (60 ug of protein) were analysed by SDS-PAGE, transferred to PDVF and blotted with anti-phospho AMPK antibody.

Figure 3.5 shows the result of immunoblotting of myocytes incubated in the presence of 25 mM glucose significantly decrease α-2 Thr172 phosphorylation compared to 0, 2, 5 mM glucose and AICAR $p < 0.005$.

 Other sites have been identified on both α and β AMPK subunits that are phosphorylated by upstream AMPKKs (Stein *et al.;* 2000). The possibility that additional, unidentified protein kinases may also regulate the activity of AMPK has also been suggested.

Figure 3.4: **The effect of glucose concentration on myocyte α-2 AMPK activity.**
Myocytes were incubated for 1 hour with the indicated concentrations of glucose followed by
freeze-stop, immunoprecipitation and assay of α-2 AMPK activity. The values are means $\pm$
S.E.M. from 7 independent myocyte preparations. A, B, C indicate $P<0.05$, <0.01, <0.005
respectively compared against zero glucose (paired tests). The significance of the paired
differences between 5 mM glucose and 2 and 25 mM glucose are shown directly on the figure.

(a)

(b)
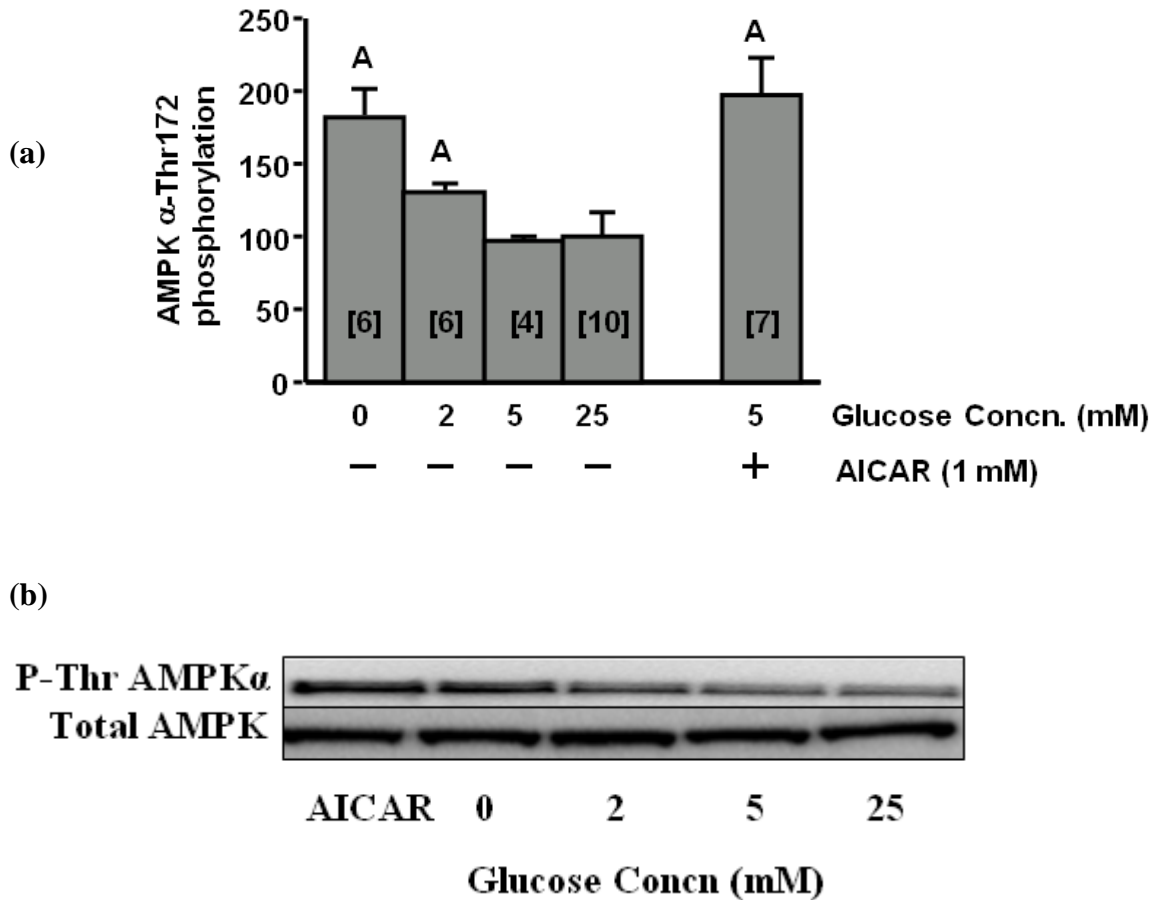
Figure 3.5: **The effect of glucose concentration and AICAR on the phosphorylation of Threonine 172 in myocyte AMPK α-subunits.** Myocytes were incubated for 1 hour with the indicated additions. (a) Phospho-AMPK measurements were normalized against measurements of total AMPK α -subunit protein and then expressed as percentages of the values in the presence of 25 mM glucose alone. The values are means ± S.E.M, of the numbers of independent myocyte preparations shown in brackets. A indicates P<0.005 compared against the 25 mM glucose value (paired tests). (b) Representitive blot.

The experiment described in **Figure 3.6** was performed in order to establish that the AMPK activity showed in Figure 3.4 was indicative of steady-state phosphorylation /dephosphorylation having being achieved. Figure 3.6 indicates that AMPK activity at 5, and 25 mM glucose was likely to have occurred after 1 hour of incubation and that then steady state persisted for at least a second hour of incubation. There were no significant differences between AMPK activities at 60, 90 or 120 min at either glucose concentration but all of these values were significantly different from zero time ($P<0.0005$, paired test). Additionally values at 30 min were significantly different (paired tests) from zero time ($P<0.025$ for 5 mM glucose and $P<0.005$ for 25 mM glucose). This is related to some experiment described later in which incubation was performed for 2 hours
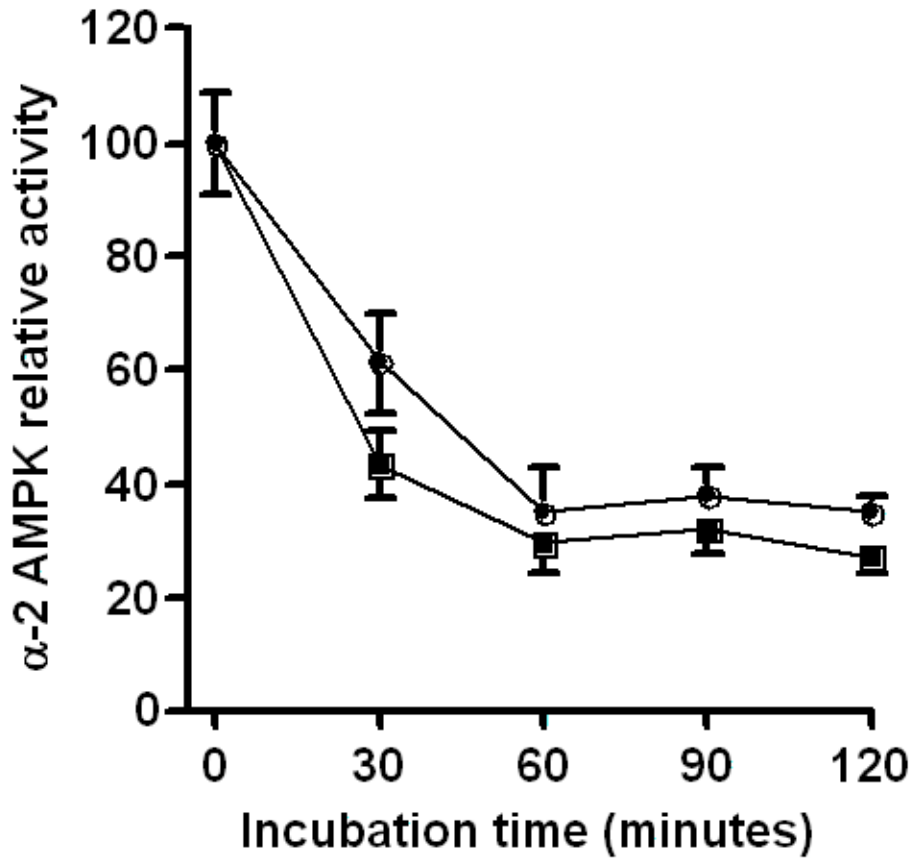
Figure 3.6: **The effect of incubation time on the ability of glucose to decrease and then stabilize myocyte α-2 AMPK activity.** Immediately after isolation myocytes were washed in glucose-free medium followed by incubation for the indicated times with either 5 mM glucose (round symbols) or 25 mM glucose (square symbols). Incubation flasks were continuously gassed with O_2/CO_2 (95:5). The values are means ± S.E.M expressed as percentages of the zero time measurements and are from 4-5 independent myocyte preparations.

3.2.3 The effect of glucose to decrease AMPK activity is not driven by changes in adenine nucleotide concentrations.

In order to determine whether the glucose –induced inactivation of AMPK was due to a decrease in cellular AMP/ATP ratio, total adenine nucleotide content was measured. Cardiac myocytes incubated with 0, 5 and 25 mM glucose without and with 0.5 mM palmitate, were subjected to acid extraction as described in **section 3.4.9** and adenine nucleotide contents were analysed using HPLC. **Table 3.1** shows ADP and AMP at 5 and 25 mM glucose were significantly different from zero. **Table 3.2** also shows 5 and 25 mM glucose in the presence of palmitate was significantly different from zero. **Figure 3.7** shows the relative ATP: ADP: AMP content at 5 and 25 Mm glucose were not significantly different from zero. Whereas cell incubated with 5 and 25 mM glucose in the presence of palmitate shows significant difference on the relative ATP:ADP:AMP content compared to the zero. .Therefore AMPK activity was determined in cells incubated with palmitate in order to see the effect of additional substrate on AMPK activity.

Figure 3.8 shows that glucose –dependent decrease in AMPK activity was still seen in the presence of palmitate and the percentage profile of this effect was very similar to that seen in the absence of palmitate **(Figure 3.4)**. It appears that the ability of glucose to decrease AMPK activity in cardiac myocytes is not due to increased ATP, and there may be a novel pathway linking glucose metabolism to the AMPK system in cardiac myocytes. Cells incubated in the absence of glucose and palmitate showed depletion in the total content of adenine nucleotides; and it is possible under this condition the activation of AMPK is due to an increase in the AMP/ATP ratio. This effect may become more significant if using myocytes incubated with no substrates are used and monitoring AMPK activity under basal conditions. Cells incubated in the absence of glucose and palmitate showed significant changes in their AMP/ATP ratio versus the cells incubated with 5 mM and 25mM glucose ($P< 0.05$ and < 0.02 respectively) **(Tables 1&2)**.
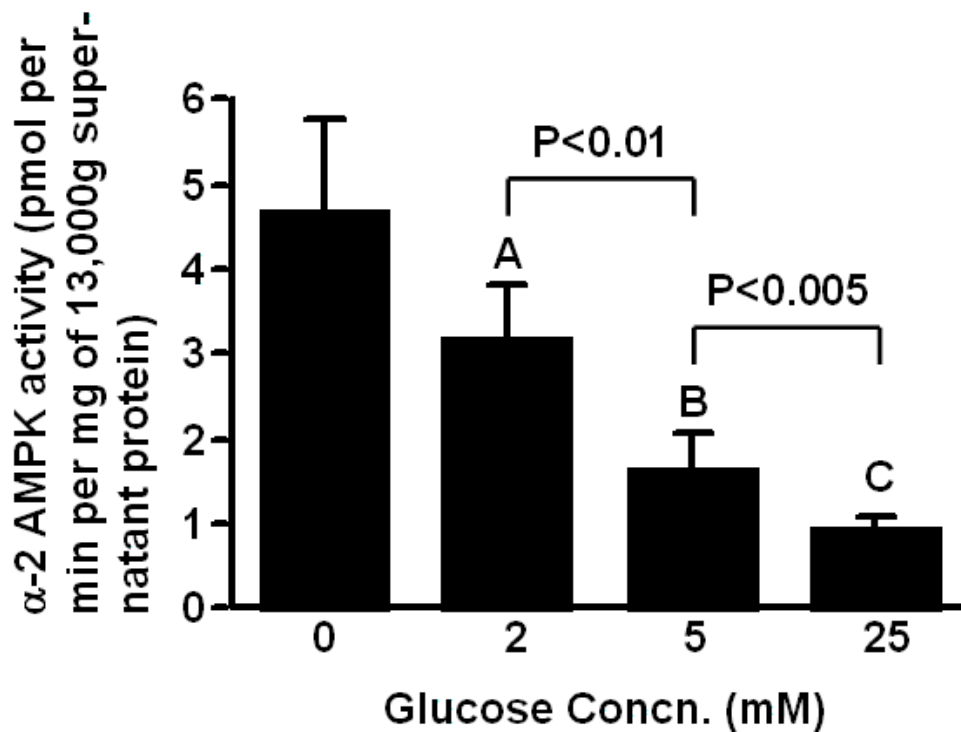
Table 3.1/ 3.2 The effect of glucose concentration on myocyte adenine nucleotide contents.

Myocytes were incubated for 1 hour with the indicated concentrations of glucose without (Table 3.1) or with 0.5 mM sodium palmitate (Table 3.2), followed by freeze-stop and measurement of ATP, ADP and AMP contents. The values are means ± S.E.M. for the numbers of independent myocyte preparations shown in brackets. The 'energy charge' was calculated from (ATP + $\frac{1}{2}$ADP)/(total adenine nucleotides).

A, B, N indicate P<0.01, <0.001, >0.05 respectively versus zero glucose (unpaired tests

Table 3.1– Incubations with glucose alone.

Adenine nucleotide contents (µmol per g wet wt. of cells)	zero glucose [8]	glucose = 5mM [7]	Glucose = 25 mM [5]
ATP	2.74 ± 0.42	8.29 ± 1.00[B]	7.86 ± 0.61[B]
ADP	2.79 ± 0.27	2.79 ± 0.53[N]	3.37 ± 0.43[N]
AMP	0.36 ± 0.08	0.39 ± 0.07[N]	0.43 ± 0.10[N]
Total adenine nucleotides ΣATP + ADP + AMP)	5.89 ± 0.61	11.47 ± 1.60[A]	11.70 ± 0.69[B]
Energy charge	0.693 ± 0.022	0.832 ± 0.029[A]	0.816 ± 0.023[A]

Table 3.2 – Incubations with glucose plus palmitate.

Adenine nucleotide contents (µmol per g wet wt. of cells)	zero glucose [7]	glucose = 5mM [5]	Glucose = 25 mM [5]
ATP	5.55 ± 0.70	5.62 ± 0.96[N]	7.70 ± 1.11[N]
ADP	3.61 ± 0.41	2.93 ± 0.58[N]	4.23 ± 0.79[N]
AMP	0.78 ± 0.13	0.57 ± 0.11[N]	0.75 ± 0.18[N]
Total adenine nucleotides ΣATP + ADP + AMP)	9.95 ± 0.90	9.12 ± 1.60[N]	12.47 ± 1.40[N]
Energy charge	0.738 ± 0.023	0.758 ± 0.027[N]	0.782 ± 0.028[N]
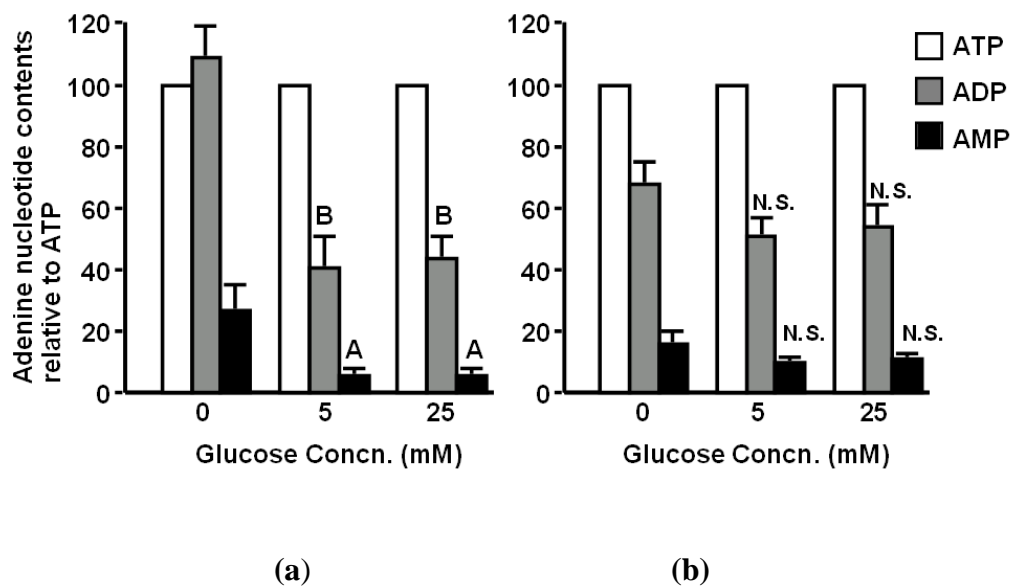
Figure 3.7: **The effects of glucose concentration and palmitate on the relative contents of ATP, ADP and AMP in myocytes.** The measurements were obtained in the experiments shown in Table 3.1/3.2. These are expressed as percentages $\pm$ S.E.M. of the ATP content. A, B indicate $P<0.05$, <0.001 respectively versus zero glucose (unpaired tests). N.S. indicates $P>0.5$ versus zero glucose.(a) glucose alone (b) glucose with palmitate
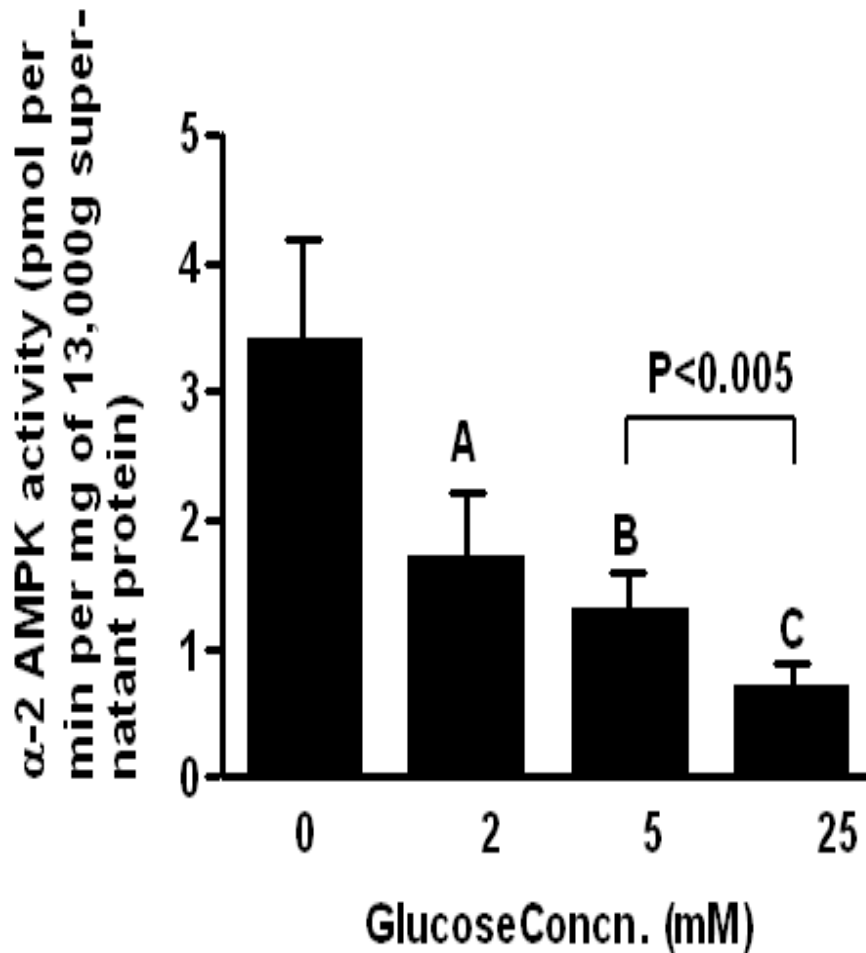
Figure 3.8: The effect of glucose concentration on myocyte α-2 AMPK activity in the presence of palmitate. Myocytes were incubated for 1 hour with 0.5 mM sodium palmitate together with the indicated concentrations of glucose followed by freeze-stop, immunoprecipitation and assay of α-2 AMPK activity. The values are means ± S.E.M. from 7 independent myocyte preparations.A, B, indicate $P<0.01$, <0.005 respectively compared against zero glucose (paired tests). The significance of the paired difference between 5 and 25 mM glucose is shown directly on the figure.

It has been reported previously that increased activation and phosphorylation of AMPK occurs under conditions where cellular AMP/ATP is increased. However as was mentioned earlier, Itani *et al;*. 2003 found that in skeletal muscle decreased AMPK activity with increasing glucose was not accompanied by changes in the concentration of adenine nucleotides or creatine-P through out the incubation period. Leptin has been shown to activate α-2 AMPK in skeletal muscle independent of the AMP: ATP ratio (Minokoshi *et al.;* 2002). Treatment of perfused rat hearts with insulin also decreased AMPK activity in aerobic hearts and prevented activation of AMPK during ischaemia without any detectable change in the AMP/ATP ratio (Beauloye *et al*.; 2001). Clark *et al.;* 2004 found that perfustion of the rat hearts with palmitate increased AMPK phosphorylation at α-2Thr172 without any change in the tissue AMP/ATP ratio.

These results suggest that glucose decreases AMPK activity independently of adenine nucleotides. The mechanism by which glucose decreases AMPK activity and phosphorylation remains to be resolved.

3.2.4 Inactivation of AMPK by glucose in the presence of AICAR

AICAR is a cell-permeable precursor of 5-aminoimidazol-4-carboxamide ribonucleoside monophosphate (ZMP). ZMP mimics the activation of AMPK by AMP. AICAR has been shown to be taken up via nucleoside transport and metabolised to ZMP by a number of mammalian tissues, including hepatocytes (Vincent *et al.;* 1991; Corton *et al.;* 1995), adipocytes (Sullivan *et al.;* 1994), human erythrocytes (Zimmermam & Deeprose, 1978), fibroblasts (Thomas *et al.;* 1981), skeletal muscle and heart tissue (Thomson *et al.;* 2009). Once synthesised within the cell, ZMP can be further phosphorylated to ZTP or metabolised to inosine monophosphate (IMP), which can be further metabolised to adenine and guanine or degraded to hypoxanthine or uric acid.

In order to investigate whether, or not the effect of glucose on AMPK was also seen under conditions where AMPK was activated by AICAR, cardiac myocytes were incubated with 3 mM glucose without AICAR and 3, 5, 10 and 25 mM glucose in the presence of AICAR.

Cardiac myocytes incubated with AICAR AMPK activity significantly decreased as glucose concentration increased. 3 mM glucose in the present of AICR increased AMPK activity by 3.7 fold while 5, 10 and 25 mM glucose increased AMPK activity by 2.0, 1.8 and 1.5 fold respectively compared to 3 mM glucose (**Figure 3.9**).

Since AICAR mimics energy stress conditions these finding further indicated that the effect of glucose on AMPK activity could be separated from an effect that is mediated by change in adenine nucleotides. In addition that finding separated the effect of glucose from that of adrenaline. Unpublished work by Dr. Tsuchiya in our laboratory has shown that AICAR abolished an adrenaline-dependent decrease in myocyte AMPK activity and phosphorylation. However it does not differentiate the effect of glucose from that of insulin. The ability of insulin to decrease myocyte AMPK activity is totally unaffected by AICAR **(see section 3.2.6).**
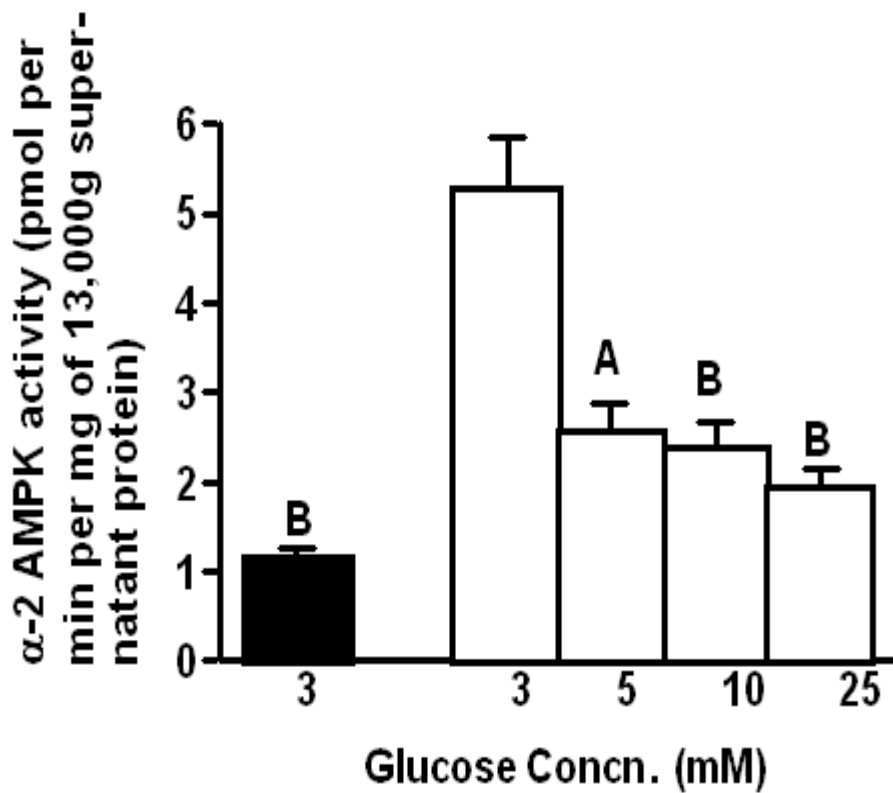
Figure 3.9: **The effect of glucose concentration on myocyte α-2 AMPK activity in the presence of AICAR.** Myocytes were incubated for 1 hour without (filled symbol) or with 1 mM AICAR (open symbols) together with the indicated concentrations of glucose followed by freeze-stop, immunoprecipitation and assay of α-2 AMPK activity. The values are means ± S.E.M. from 4 independent myocyte preparations. A, B indicate $P<0.01$, <0.005 respectively compared against 3 mM glucose with AICAR (paired tests).

3.2.5 Conclusion

AMPK activity decreases as glucose concentration increases in cardiac myocytes incubated for 1 hour and the decrease on AMPK activity correlated with a decrease in the phosphorylation of α-Thr172. Incubation with the pharmacological agent AICAR had no effect on the ability of glucose to decrease AMPK activity.

As already noted the classic view is that AMPK is activated by changes in the energy states of the cell, as reflected by the AMP/ATP ratio. An increase in AMP/ATP ratio can activate AMPK allosterically and inhibit its dephosphorylation by protein phosphatases. In the present study no change in adenine nucleotide contents was observed after 1 hour incubation with 5 or 25 mM glucose.

AMPK activity was increased on the incubation without glucose. Therefore note that this is a totally abnormal state as total adenine nucleotides were decreased suggesting that the cells were so sick that they were catabolising their adenine nucleotides.

Figure 3.10 shows that percentage effect of glucose on AMPK activity in the presence and absence of palmitate were broadly similar. For comparison the percentage effect of glucose in the presence of AICAR is also shown.
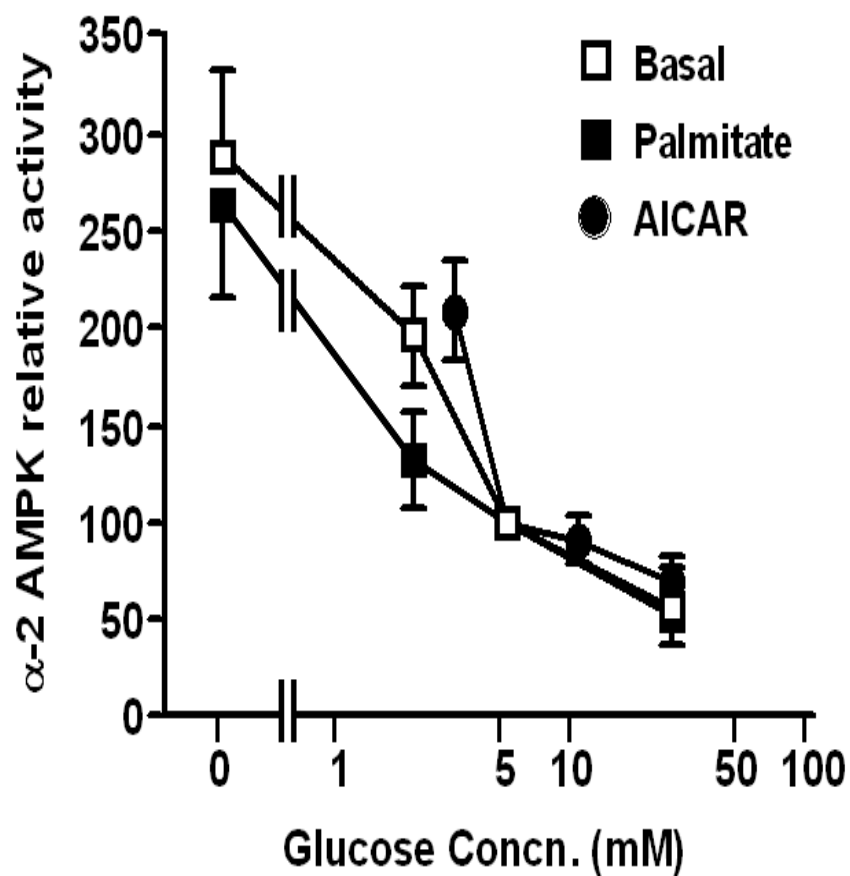
Figure 3.10: Summary of the effects of glucose concentration on myocyte α-2 AMPK activity. The values from the experiments shown in Figures 3.4, 3.8 and 3.9 are shown expressed as percentages of measurements with 5 mM glucose. The bars indicate S.E.M.

3.2.6 Interactions between AMPK activity, insulin, adrenaline and glucose

Other work has shown that insulin and adrenaline can both increase glucose uptake by myocytes and can decrease AMPK activity/phosphorylation. Therefore it was necessary to investigate whether the effect of glucose was totally independent of the effect of insulin (mediated through PKB) or the effect of adrenaline (via an unknown signalling pathway, which is independent of PKB) or whether there was some overlap of the glucose effect with either of these signalling pathways. In perfused hearts, insulin has been shown to decrease the basal AMPK activity as well as the activation of AMPK during ischemia. The mechanism of this inhibition is not fully understood (Beauloye *et al.;* 2001). The effect of insulin has been reported to be dependent on PI3K activity, but independent of changes in the AMP/ATP or Cr/CrP ratios (Beauloye *et al.;* 2001). Previous studies have illustrated the involvement of PKB and the inhibitory effect of insulin on AMPK activity (Horman *et al.;* 2006). Kovacic *et al.* (2003) have shown that the effect of insulin on AMPK activity is mimicked by overexpressing constitutively active PKB, suggesting an involvement of the PI3K-PKB pathway (Kovacic *et al.;* 2003). The effect of insulin on AMPK activity was examined in cardiac myocytes in order to establish whether there is any link in the mechanisms by which the sugar and the hormone decrease AMPK activity.

3.2.6.1 Effect of insulin on AMPK activity

Figure 3.11 shows that 5 mM glucose decreased AMPK activity by 82% in both the absence and presence of insulin and insulin decreased AMPK activity by 31% and by 33% in the absence and presence of 5 mM glucose respectively. In Experiment B increasing glucose from 5 to 25 mM decreased AMPK activity by 45% and by 41% in the absence and presence of insulin respectively and insulin decreased AMPK activity by 44% and by 40% with 5 mM and 25 mM glucose respectively.
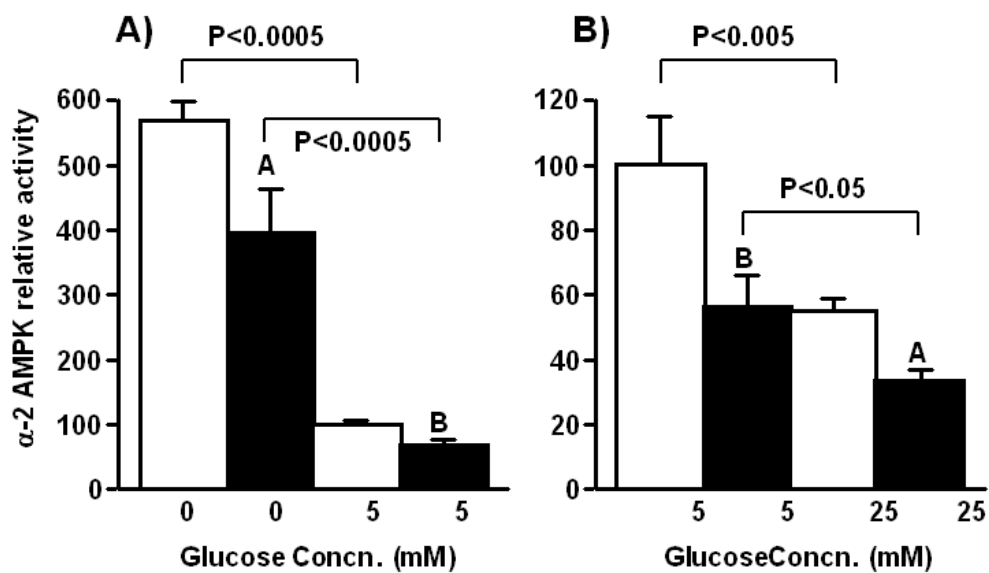
Figure 3.11: The effects of glucose and insulin to decrease myocyte α-2 AMPK activity are independent. The figure is a combination of two separate experiments (A & B). In both cases myocytes were incubated for 1 hour with the indicated concentrations of glucose without (open symbols) or with 10 nM insulin (filled symbols). The values are expressed as means ± S.E.M. of the measurements with 5 mM glucose alone. 6 independent myocyte preparations were used in Experiment A and 4 separate preparations were used in Experiment B. A, B indicate $P<0.05$, <0.025 respectively for effects of insulin (paired tests). Paired test P values for effects of glucose are shown directly on the figure.

3.2.6.2 The effect of glucose concentration on the phosphorylation state of Serines 636/639 in myocyte insulin receptor substrate-1 (IRS-1)

In general, reduced IRS activity is associated with reduced tyrosine phosphorylation and/or increased serine phosphorylation. To determine levels of IRS1 phosphorylation of Ser636/639, cells were incubated with zero or varying glucose concentrations. **Figure 3.12** shows that IRS-1 phosphorylation levels at 2, 5, and 25 mM glucose were not significantly different when compared to zero glucose. These results suggested that IRS-1 was not involved in the signalling pathway whereas glucose decreases AMPK activity.

The experiment shown in **Figure 3.13** was performed as a control to accompany **Figure 3.12**; ie it shows that IRS-1 phosphorylation responded normally to insulin. Interestingly AICAR in the presence of insulin slightly enhanced the phosphorylation of IRS1 Ser636/639 in response to insulin. A, B indicate $P<0.025$, <0.005 respectively for effects of insulin versus the appropriate control (paired tests). The P value shown directly on the figure indicates the significance of the effect of AICAR in the presence of insulin (paired test). AICAR had no significant effect on IRS-1 phosphorylation in the absence of insulin.

(a)

(b)
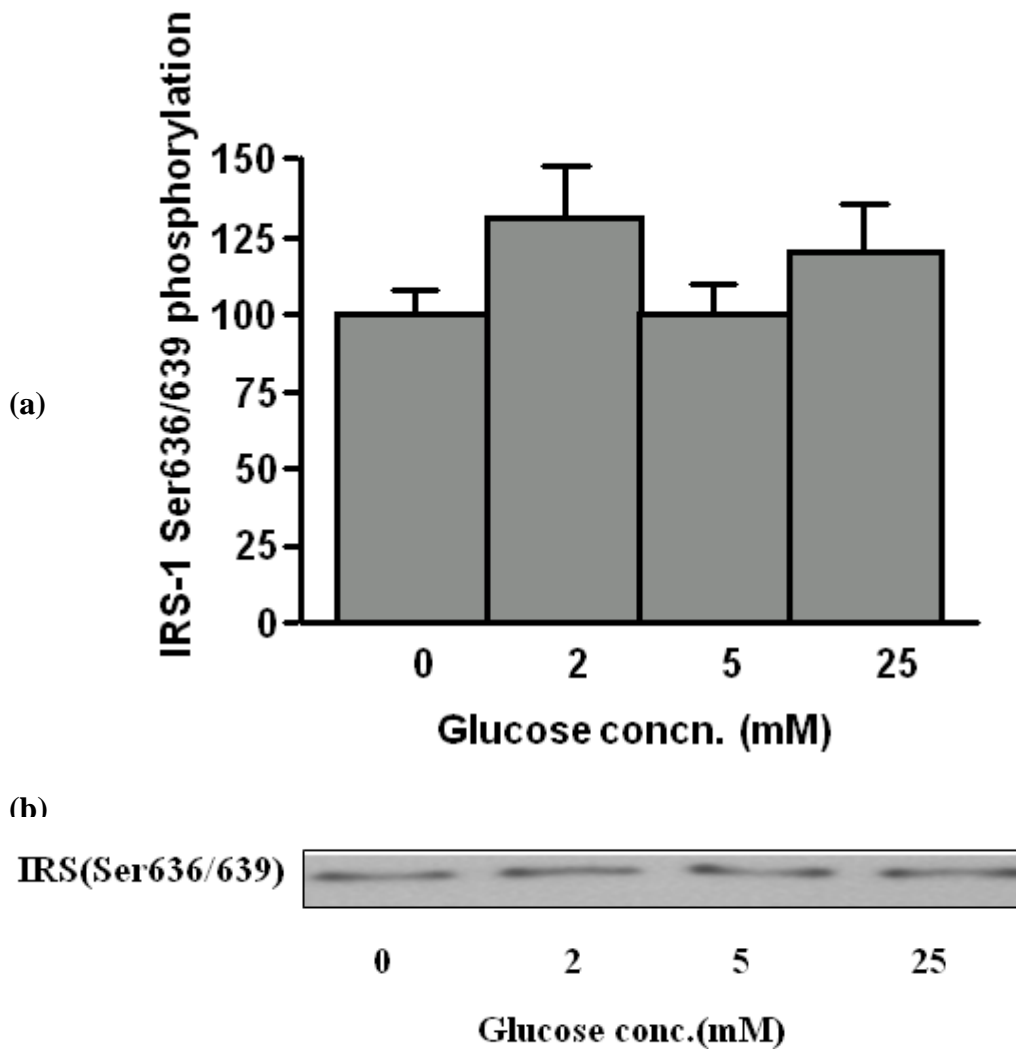
Figure 3.12: **the effect of glucose concentration on the phosphorylation state of Serines 636/639 in myocyte insulin receptor substrate-1 (IRS-1)**. Myocytes were incubated for 1 hour with the indicated concentrations of glucose. (a)Phospho-IRS-1 measurements were normalized against measurements of total IRS-1 protein and then expressed as percentages of the values in the presence of 5 mM glucose. The values are means ± S.E.M. from 5 independent myocyte preparations. No significant effects of glucose were observed. (b) Representitive blot.

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(a)

(b)
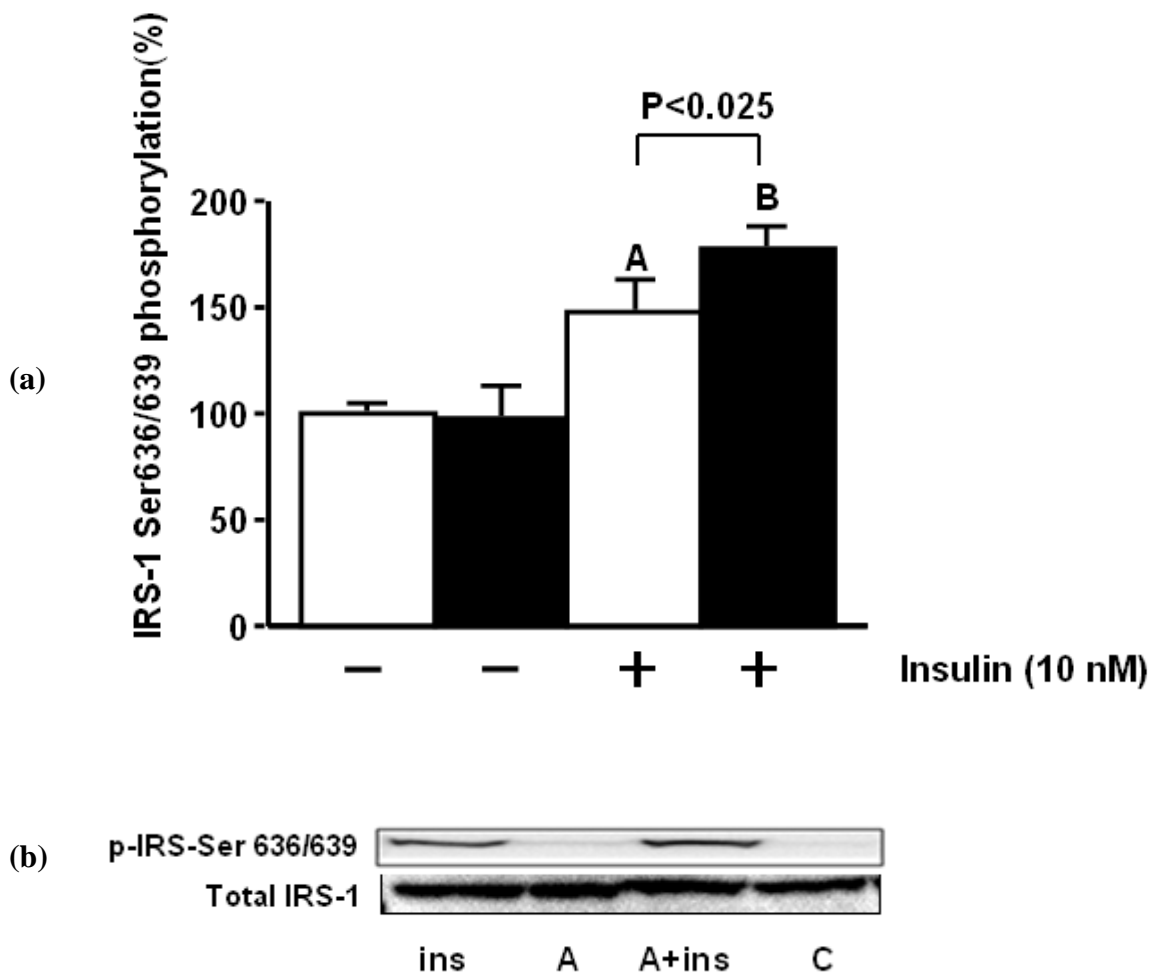
Figure 3.13: **AICAR enhanced the effect of insulin on the phosphorylation state of Serines 636/639 in myocyte insulin receptor substrate-1 (IRS-1**). Myocytes were incubated for 1 hour with 5 mM glucose without (c) (open symbols) or with 1 mM AICAR(A) (filled symbols). Insulin was added as indicated (ins) or (ins+A). Phospho-IRS-1(a) measurements were normalized against measurements of total IRS-1 protein and then expressed as percentages of the values in the absence of AICAR and insulin. The values are means ± S.E.M. from 5 independent myocyte preparations. (b) Representative blot.

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3.2.6.3 Variation of glucose concentration had no effect upon the phosphorylation state of myocyte protein kinase B (PKB).

PKB is known to mediate a number of biological responses to insulin and growth factors in various tissues, its role in glucose uptake being one of the most extensively studied. PKB activity is required for the insulin-induced activation of phosphodiesterase 3B (PDE3B), and for the antilipolytic action of insulin in adipocytes. Moreover inhibition of PKB activity resulted in reduction in de novo lipid synthesis and in the ability of insulin to stimulate this process in lipogenic tissues. The regulation of the rate-limiting enzyme acetyl-CoA carboxylase (ACC) by insulin through dephosphorylation of AMPK phosphorylation site was dependent on the presence of active PKB. It is also required for positive effect of insulin on lipid storage in adipocytes. Next in this study the effect of glucose on myocyte PKB was examined in order to determine whether PKB could mediate the glucose effect on AMPK.

Figure 3.14 shows that glucose at 0, 2, 5 and 25 mM glucose had no significant effect on PKB Thr 308 and Ser 473 phosphorylation. As expected insulin as a control significantly increased the phoshorylation of PKB at both Thr 308 and the Ser 473 sites ($p<0.005$).

3.2.6.4 The effect of glucose and insulin on phosphorylation of AMPK α-subunit Ser485/491

Recently, Horman *et al.* 2006 demonstrated that the AMPK α subunit is phosphorylated at Ser485 (α1) or Ser491 (α2) by PKB *in vitro* or in response to insulin in perfused hearts. The phosphorylation of α- Ser485/491 was shown to inhibit the phosphorylation of α- Thr172 and this was suggested to be the mechanism by which insulin inhibits AMPK (Horman *et al.,* 2006). The effect of glucose on α- Ser485/491 phosphorylation of AMPK was examined using a commercially available antibody. This antibody was previously used successfully to detect an increase in α- Ser485/491 phosphorylation by PKB (Soltys *et al.;* 2006).
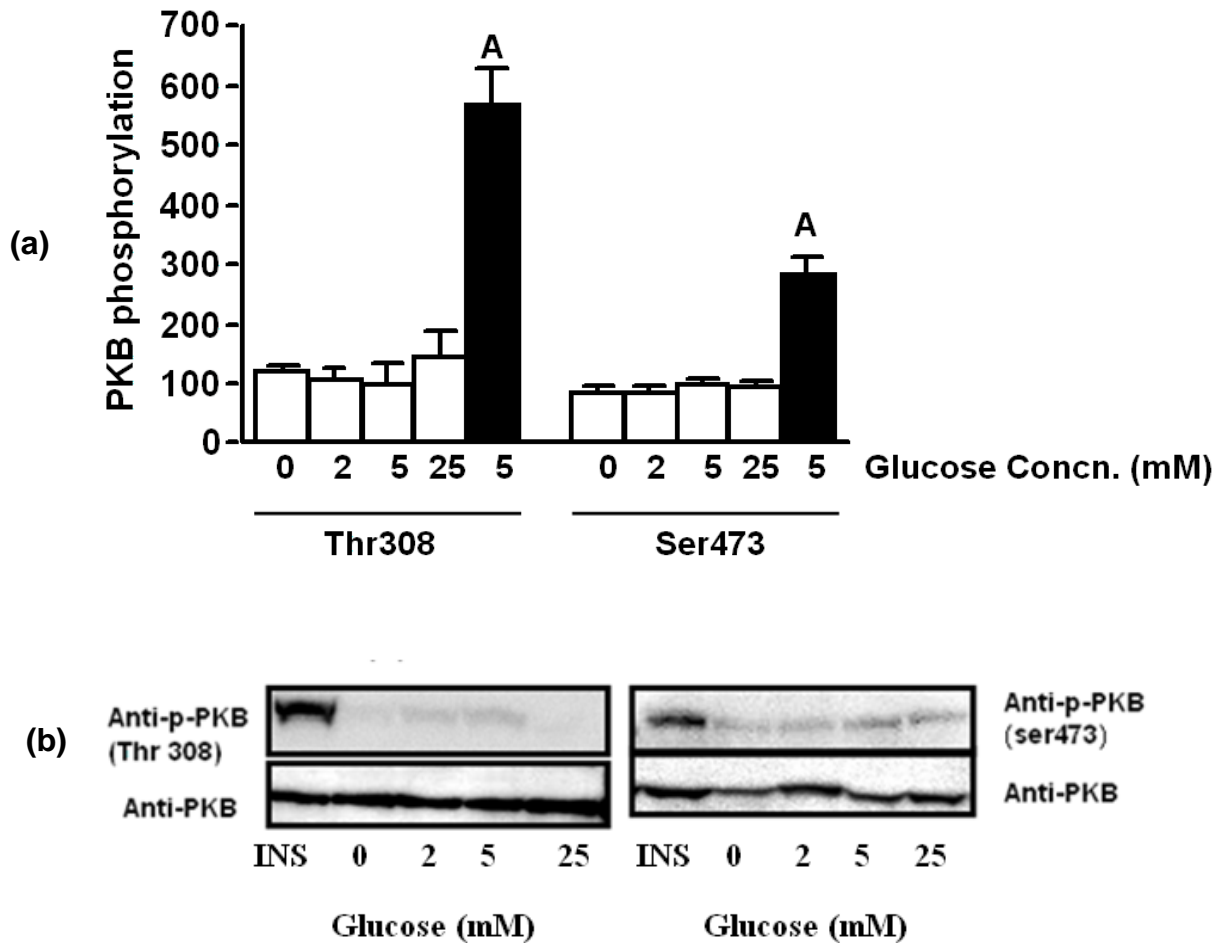
Figure 3. 14: **Variation of glucose concentration had no effect upon the phosphorylation state of myocyte protein kinase B (PKB).** **(a)** Myocytes were incubated for 1 hour with the indicated concentrations of glucose without (open symbols) or with 10 nM insulin (filled symbols). Phospho-PKB measurements were normalized against total PKB protein and then expressed as percentages of the values in the presence of 5 mM glucose alone. The values are means ± S.E.M. for 4 independent myocyte preparations. A indicates P<0.005 for the effects of insulin at 5 mM glucose (paired tests) (b) Representitive blot

Figure 3.15 Cardiac myocytes incubated with 0, 2, 5 and 25 mM glucose showed no appreciable difference in Ser485/491 phosphorylation, whereas in cells incubated with insulin it increased the phosphorylation of Ser485/491 compared to the 0, 2, 5 and 25 mM glucose. This result confirms previous work (Horman *et al*.; 2006) in that insulin was able to increase the phosphorylation of AMPK α-Ser485/491 at 5 mM glucose (result from 3 independent myocyte preparations). However, in a single preparation glucose alone has no noticeable effect on α- Ser 485/491 phosphorylation.
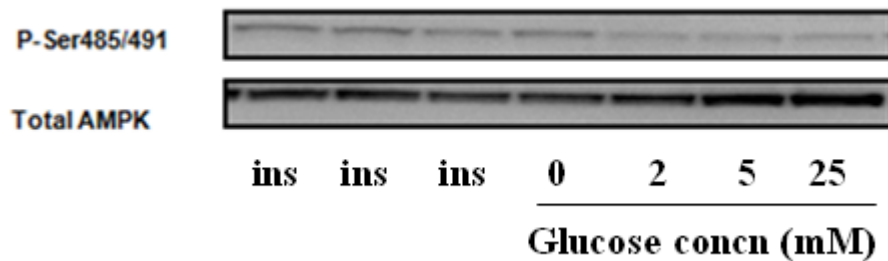
Figure 3.15: **The effect of glucose on AMPK Ser485/491 phosphorylation.**
Phosphorylation of AMPK Ser485/491 was examined after incubating cardiac myocytes for 1 hour with 0, 2, 5, 25 mM glucose or 5 mM glucose in the presence of 10nM insulin (ins). Cell lysates were used for Western blot analysis. Membranes were probed with AMPK Ser 485/491 phospho and AMPK total antibody. The figures show results from four independent myocytes preparations.

3.2.6.5 The effect of AICAR to enhance the effect of insulin on phosphorylation of PKB

Figue 3.16 shows a significant increase in the phosphorylation of Thr 308 and Ser 473 in the presence of insulin compared to the control. Surprisingly AICAR in the presence of insulin caused a significant increase in PKB phosphorylation at Thr 308 and Ser 473 compared to when cells were incubated with insulin alone ($p<0.025$, $p<0.05$ respectively). This result suggests that during hyperglycemic conditions glucose might play a part to antagonise PKB activity. Therefore the activation of AMPK might be beneficial during metabolic stress.

3.2.6.6 The effect of insulin on AMPK activity on the presence of AICAR or oligomycin

In previous experiments, high glucose was shown to decrease AMPK activity in the presence of AICAR. It was additionally investigated whether the effect of insulin could be maintained under conditions where AMPK was activated by other AMPK-activating stimuli; i.e. AICAR or oligomycin. Cardiac myocytes were incubated with insulin in the presence of AICAR or oligomycin for 1 hour and then AMPK activity was examined under these conditions.

Figure 3.17 shows AICAR and oligomycin significantly increased AMPK activity ($p<0.01$, p<0 05). However the effect of insulin on AMPK activity was totally preserved in the presence of these agents; i.e. the percentage decreases in AMPK activity due to insulin were 44% without AICAR or oligomycin, 46% in the presence of AICAR and 48% in the presence of oligomyocin.

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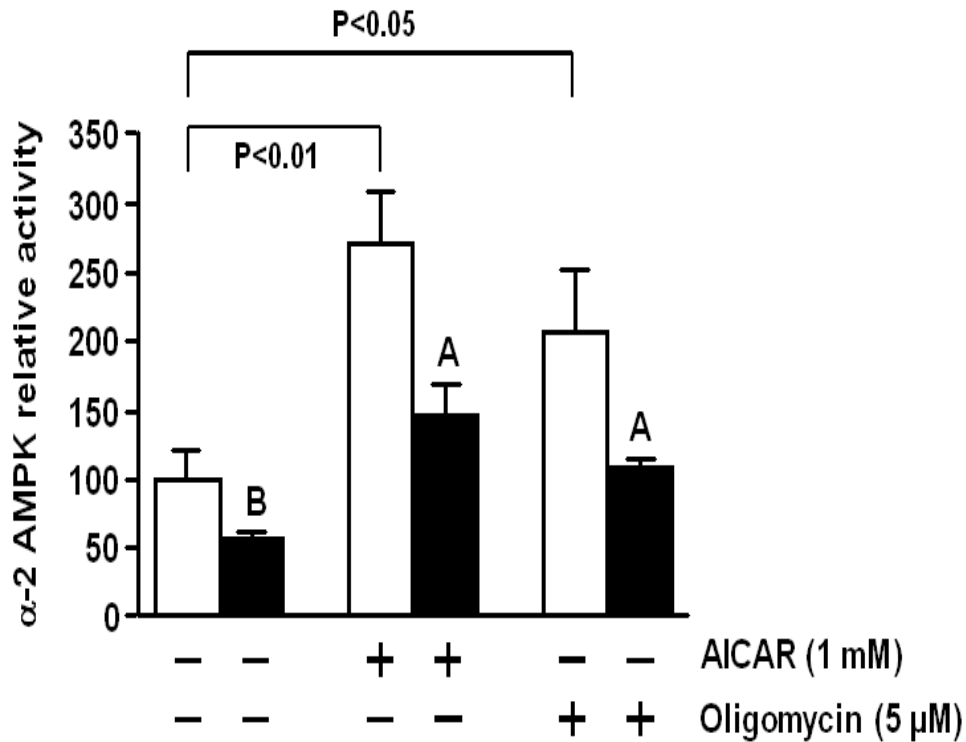
Figure 3.16: **The effects of insulin on myocyte α-2 AMPK activity in the presence of AICAR or oligomycin.** Myocytes were incubated for 1 hour with 5 mM glucose without (open symbols) or with 10 nM insulin (filled symbols) together with other additions as indicated. The values are means ± S.E.M. of the measurements with 5 mM glucose alone and are from 5 independent myocyte preparations. A, B indicate $P<0.01$, <0.005 respectively for effects of insulin (paired tests). Paired test P values for effects of AICAR and oligomycin are shown directly on the figure.

(a)

(b)
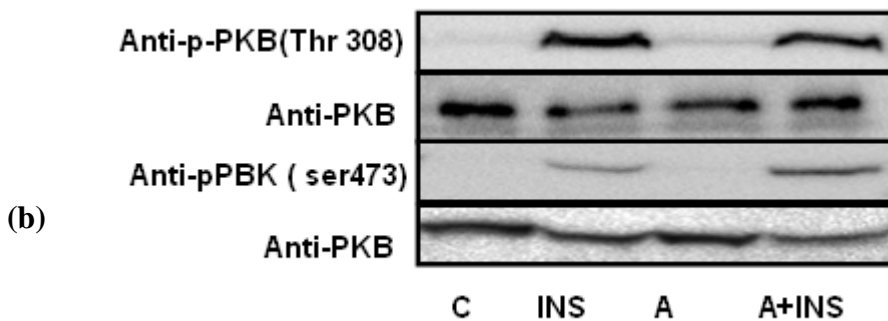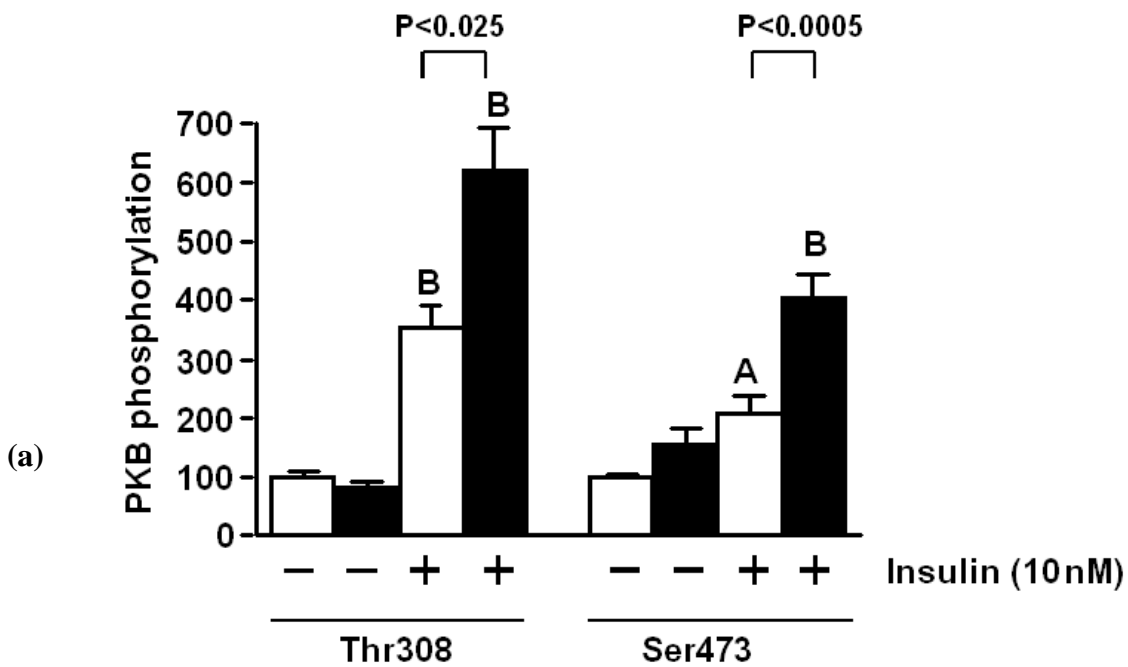
Figure 3.17: **AICAR enhanced the effect of insulin on the phosphorylation state of myocyte protein kinase B (PKB).** (a)Myocytes were incubated for 1 hour with 5 mM glucose without (open symbols) or with 1 mM AICAR (filled symbols).10 nM insulin was added as indicated. Phospho-PKB measurements were normalized against total PKB protein and then expressed as percentages of the values in the absence of insulin and AICAR. The values are means ± S.E.M. for 5 independent myocyte preparations. A, B indicate P<0.025, <0.005 respectively for effects of insulin versus the appropriate control (paired tests). Paired P values shown directly on the figure indicate the significance of effects of AICAR in the presence of insulin. AICAR had no significant effect on PKB phosphorylation in the absence of insulin.(b) representititive blot

3.2.7 The effect of an inositol phosphoglycan (IPG) preparation on AMPK activity

It was previously reported that ceramide mediated the inhibition of AMPK by palmitate through the activation of PP2A (Yong *et al.;* 2007). The action of extracellular agonists results in the activation of a variety of lipases. This causes the generation of lipid-derived second messengers, which comprise a growing list of molecules, including inositol (1,4,5)-trisphosphate, diacylglycerol, ceramide, lysophosphatidic acid, and others (Mato, 1990; Divecha & Irvine, 1995). Inositol phosphoglycan (IPG) insulin second messenger, which has been reported to have potential effect to lower blood glucose in diabetic rats (Ikhlass & Gumma Patent no WO/2000/039135) and mimics the short-and long-term actions of insulin (Romero & Lamer, 1993, Romero *et al.;* 1988), could perhaps mediate the effect of glucose on AMPK activity. In order to test this, cardiac myocytes were incubated with 5 and 25 mM glucose in the presence or absence of 10 μg/ml IPG (personal contact). **Figur**e **3.18** shows that with myocytes incubated with 5 or 25 mM glucose IPG preparation significantly increased AMPK activity to the extent that the difference between AMPK activites at 5 and 25 mM glucose was a bolished . This possibly suggests that IPG preparation work in vivo as AMPK activator. If it is an AMPK activator in the liver this could leads to inhibition of gluconeogenesis and a lowering of blood glucose. However the effect of this preparation were not investigated further, including the possibility that it may have increased myocyte AMPK activity through increase cellular AMP/ATP ratio.

3.2.8 The effect of glucose on AMPK activity was severely blunted in the presence of adrenaline.

Figure 3.19 shows that, in the presence of adrenaline, variation of glucose concentration in the range 0 to 15 mM had no significant effect upon α-AMPK activity. 5 mM glucose was used in this experiment as a control because previous work in our laboratory has showed that adrenaline

decreased AMPK activity at the same glucose concentration. The results provide the expected effect which is that adrenaline significantly decreased AMPK activity. It has been reported that, in perfused hearts, adrenaline may antagonise the activation of α2 AMPK by palmitate (Clark *et al..;* 2004). The stimulation of heart tissue with adrenaline is associated with a change in fuel selection, adrenaline increases the rate of ATP production as the energy needs of the heart increases; it does this by increasing both glucose and fatty acid oxidation (Collins *et al.;* 1994).
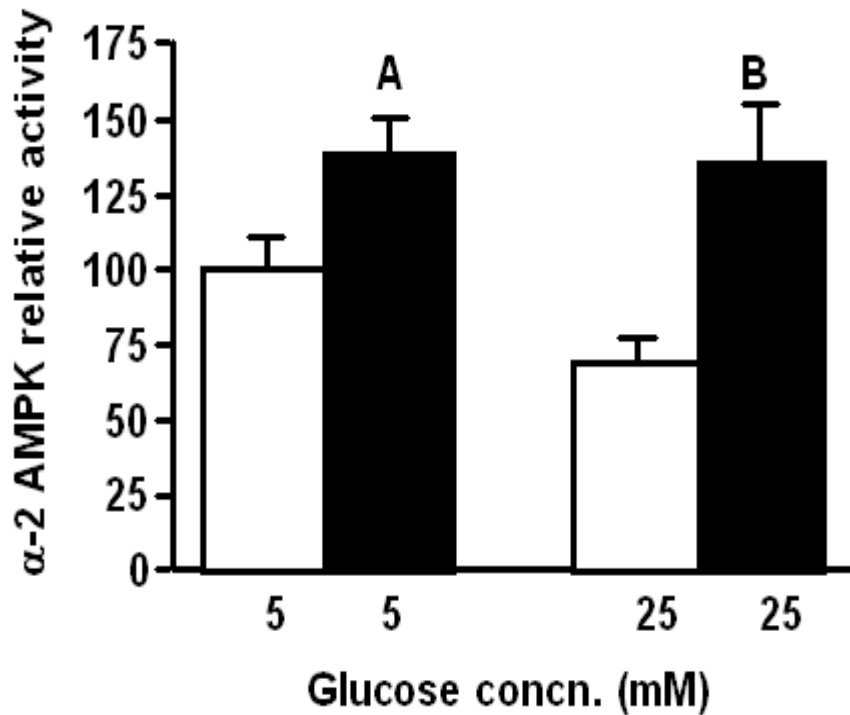
Figure 3.18: The effect of an IPG preparation on myocyte α-2 AMPK activity.

Myocytes were incubated for 1 hour with the indicated concentrations of glucose without (open symbols) or with 10 μg/ml of IPG (filled symbols). The values are means ± S.E.M. from 6 independent myocyte preparations expressed as percentages of the measurements in the presence of 5 mM glucose alone. A, B indicate P<0.05, <0.025 for effects of IPG versus the appropriate control (paired tests).
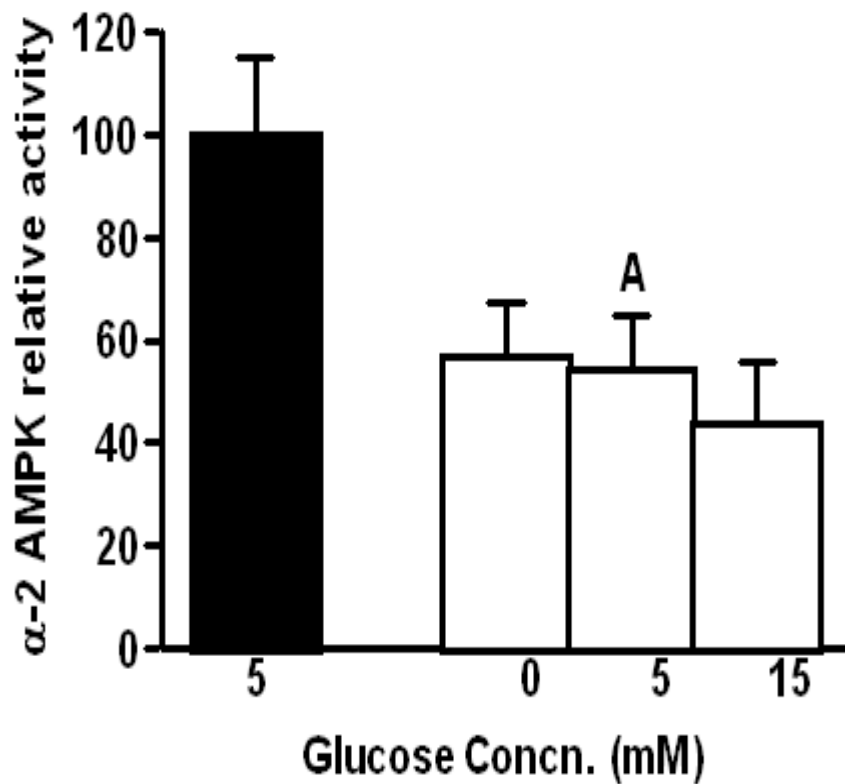
Figure 3.19: **Attenuation by adrenaline of the effect of glucose upon myocyte α-2 AMPK activity.** Myocytes were incubated for 1 hour with the indicated concentrations of glucose without (filled symbol) or with 2 µM adrenaline (open symbols). The values are means ± S.E.M. expressed as percentages of the measurements with 5 mM glucose alone and are from 5 independent myocyte preparations.A indicates P<0.01 for the effect of adrenaline at 5 mM glucose (paired test).

3.2.9 Conclusion

Glucose had no effet on phosphorylation of IRS-I Ser363/639, PKB Ser308, Thr473 and AMPK Ser485/491. These results suggested that glucose decreases AMPK activity independently of insulin. The other finding that AICAR enhances the phosphorylation of PKB Ser308 and Thr473 in the presence of insulin strengthens the hypothesis that AMPK could be a therapeutic target for type-2 diabetes.

Interestingly an IPG preparation which, has been reported to decrease glucose levels in diabetic rats increased AMPK activity. The mechanism by which IPG preparation increased AMPK activity is unclear.

Adrenaline has been reported to decrease cardiac myocyte AMPK activity by a mechanism which is not understood. In unpublished data from our laboratory showed that the effect of adrenaline on AMPK activity was completely blocked in the presence of AICAR. Insulin and glucose were found to decrease AMPK activity in the presence of AICAR. Therefore these results suggest that insulin, glucose and adrenaline decrease AMPK activity through distinct mechanisms.

Section: 3

3.3 The effect of increasing glucose concentration on AMPK phosphorylation targets in myocytes.

3.3.1 Acetyl- CoA Carboxylase

AMP-activated protein kinase (AMPK) is activated during muscle contraction in response to an increase in AMP. Once activated, AMPK has been proposed to phosphorylate a number of targets, resulting in increases in glucose transport, fatty acid oxidation, and gene transcription. It has been reported that using a phosphoserine antibody to ACC and a phosphothreonine antibody to AMPK, evidence was obtained for phosphorylation and activation of ACC in vitro in gastrocnemius muscle electrically stimulated at different frequencies, and in muscle from rats running on a treadmill. Significant negative linear correlations between phospho-ACC and ACC activity were observed. (Park *et al.;* 2002) In heart AMPK is able to phosphorylate ACCα and ACCβ, resulting in an almost complete loss of ACC activity. ACC in the heart exist in a heterodimeric structure, and that this structure is tightly associated with the α_2 subunit of AMPK. (Saha *et al.;* 2001). As ACC is a well established downstream target of AMPK in heart and other cell types, in the present study glucose decreased AMPK activity and this might has impact on ACC phosphorylation.

Figure 3.20 shows that, relative to incubation at 5 mM glucose ACC Ser227 phosphorylation was significantly higher at zero and 2 mM glucose and was significantly lower at 25 mM glucose. The decrease in ACC phosphorylation between 5 and 25 mM glucose (without insulin) was very similar to the decrease in ACC phosphorylation when insulin was added to myocytes at 5 mM glucose. The effect of AICAR upon the phosphorylation of ACC at 5 mM glucose was also measured by way of comparison.

Figure 3.21 this experiment demonstrates that increasing glucose concentration decreased ACC phosphorylation in the presence of AICAR. This experiment complements that in **Figure 3.9** which showed that AICAR did not abolish the effect of glucose to decrease AMPK activity.

Figure 3. 22 shows that insulin significantly decreased the phosphorylation of ACC Ser277 by AICAR or oligomyocin. These decreases on ACC Ser227 phosphorylation correlated with a decrease on AMPK activity. Therefore, it appears that glucose and insulin have genuine direct inhibitory effect on AMPK in cardiac myocytes.

Figure 3.23 shows a positive linear correlation between changes in the phosphorylation of Serine 227 in ACC and changes α -2 AMPK activity in cardiac myocytes.

(a)

(b)
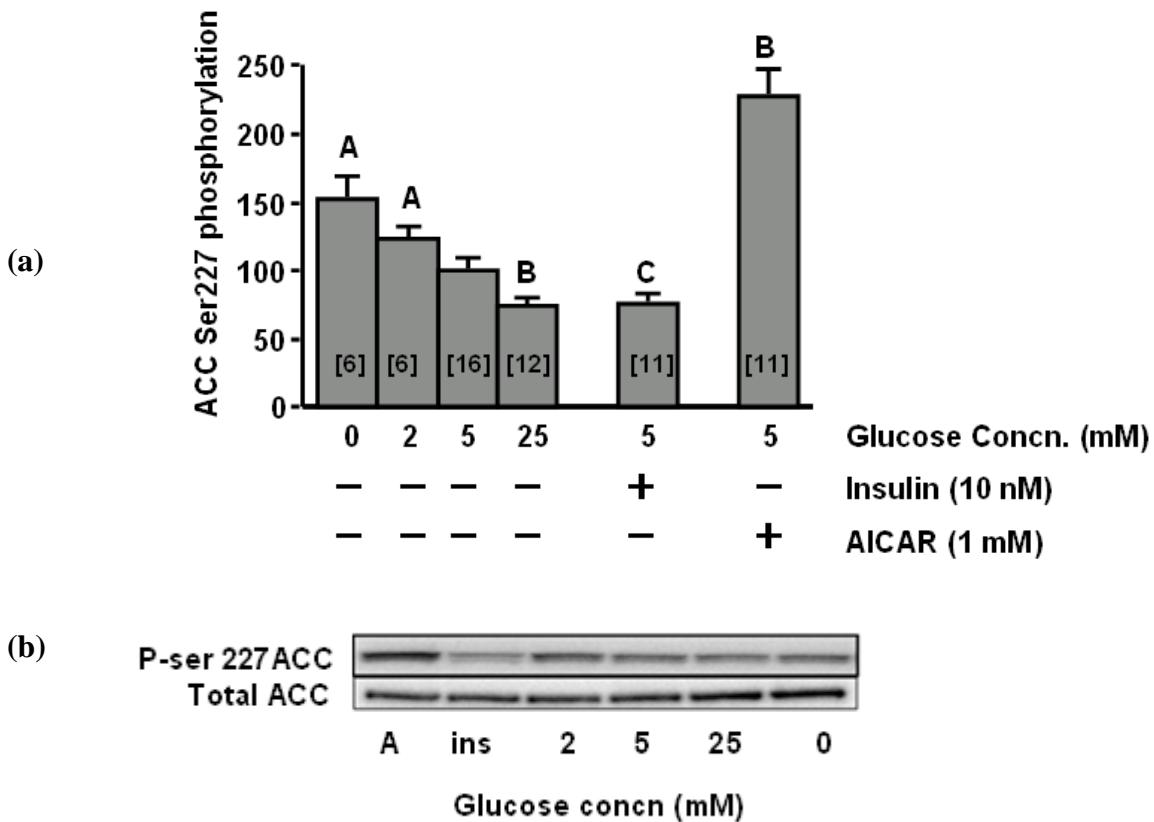
Figure 3.20: **The effect of glucose concentration, insulin and AICAR on the phosphorylation of Serine 227 in myocyte acetyl-CoA carboxylase** (ACC). (a)Myocytes were incubated for 1 hour with the indicated additions. Phospho-ACC measurements were normalized against measurements of total ACC protein and then expressed as percentages of the values in the presence of 5 mM glucose alone. The values are means ± S.E.M. of the numbers of independent myocyte preparations shown in brackets. A, B, C indicate P<0.025, <0.01, <0.005 respectively compared against the 5 mM glucose value (paired tests).(b) Representitive blot.
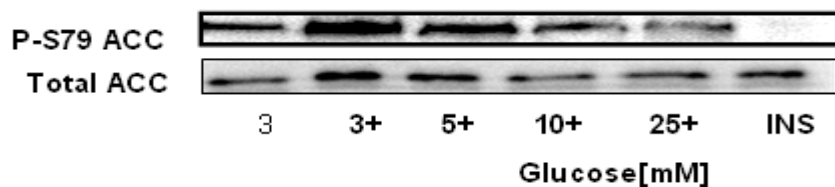
Figure 3.21: **Effect of glucose on ACCS Ser227 phosphorylation in the presence of AICAR**. Cardiac myocytes were incubated at the indicated glucose concentrations with or without 1mM AICAR (+) or with 10 nM insulin (INS). Cells were lysed and analysed by western blotting using anti-phospho-Ser227 and total ACC antibody.
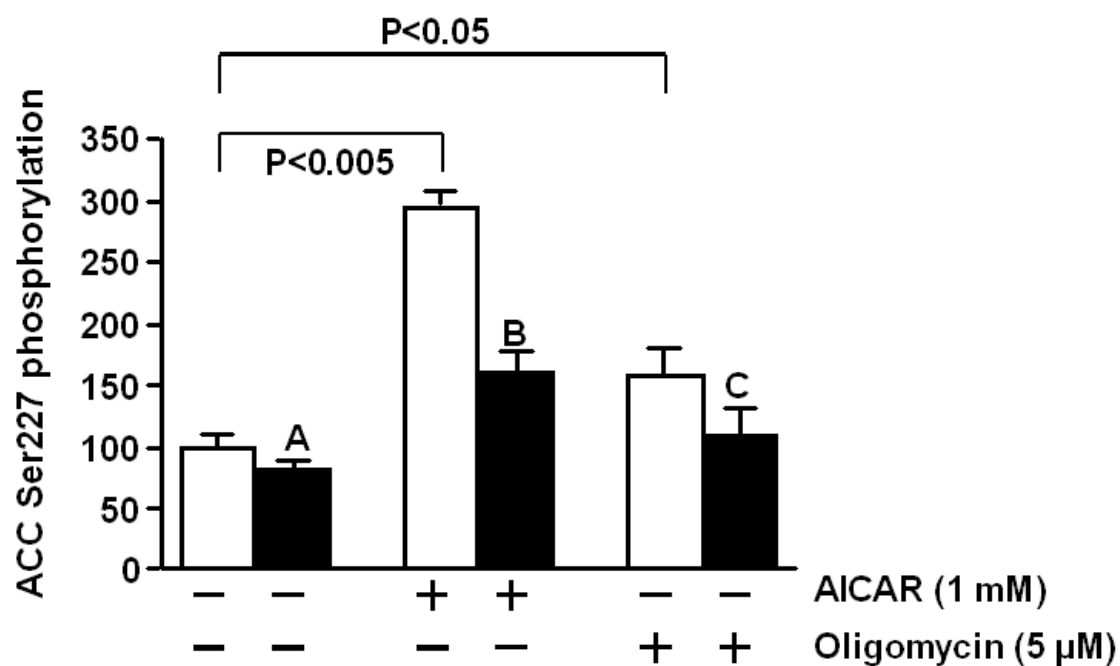
Figure 3.22: **Effects of insulin on the phosphorylation of Serine 227 in myocyte acetyl-CoA carboxylase (ACC) in the presence of AICAR or oligomycin.** Myocytes were incubated for 1 hour with 5 mM glucose without (open symbols) or with 10 nM insulin (filled symbols) together with other additions as indicated. Phospho-ACC measurements were normalized against measurements of total ACC protein. Values are means ± S.E.M. of the measurements with 5 mM glucose alone and are from the same 5 independent myocyte preparations used in the experiment shown in **Figure 3.18**.A, B, C indicate P<0.05, <0.025, <0.005 respectively for effects of insulin (paired tests). Paired test P values for effects of AICAR and oligomycin are shown directly on the figure.
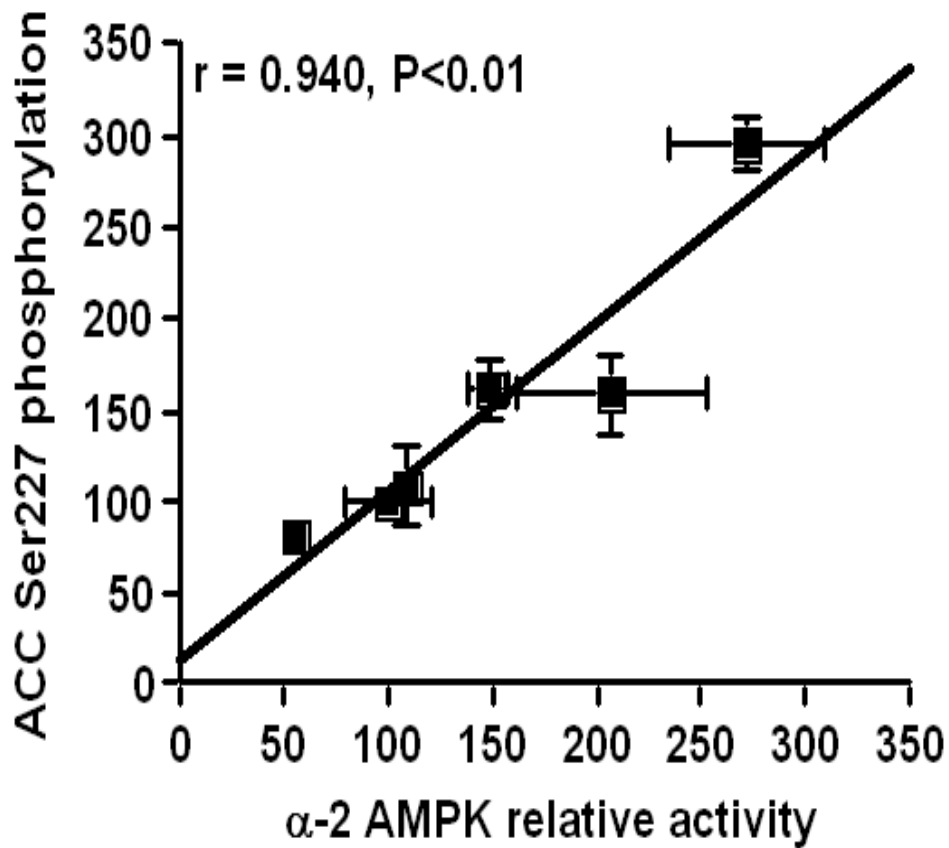
Figure 3.23: **A positive linear correlation between the phosphorylation of Serine 227 in acetyl-CoA carboxylase and α -2 AMPK activity in myocytes.** The Values are taken from Figures **3.17** and **3.22.** The bars indicate S.E.M. Where these are not shown, they lie within the symbol. The gradient of the regression line ± its standard error was 0.922 ± 0.168 which is not significantly different from a gradient of 1.0 (P<0.3).

3.3.2 The effect of glucose on PFK2 activity and the content of Fructose 2, 6-bisphosphate in cardiac myocyte

PFK-2 is an enzyme responsible for synthesis of fructose 2, 6-bisphosphate which is a powerful activator of PFK-1 in the glycolytic pathway (Toyoda *et al.*, 2004). It has been reported to be phosphorylated and activated by AMPK (Hue *et al*.; 2000). Heart PFK-2 was phosphorylated on Ser466 and activated by AMPK *in vitro*. In perfused rat hearts, anaerobic conditions or inhibitors of oxidative phosphorylation (oligomycin and antimycin) caused induced AMPK activation, which correlated with PFK-2 activation and with an increase in fructose 2, 6-bisphosphate concentration. Moreover, in cultured cells transfected with heart PFK-2, oligomycin treatment resulted in a parallel activation of endogenous AMPK and PFK-2. In these cells, the activation of PFK-2 was due to the phosphorylation of Ser466. A dominant-negative construct of AMPK abolished the activation of endogenous and cotransfected AMPK, and prevented both the activation and phosphorylation of transfected PFK-2 by oligomycin (Hue *et al.;* 2000).

 PFK-2 activity was tested in this study. The main purpose was to see what effect of varying glucose concentration had on myocyte PFK-2 activity on the basis that heart PFK-2 is activated on being phosphorylated by AMPK. Whilst glucose decreases AMPK activity the expectation was that increasing glucose concentration might decrease PFK-2 activity.PFK-2 activity was measured as described in **section 1.12.2.** However **Figure 3.24** shows a significant increase in PFK-2 activity at 2, 5, and 25 mM glucose compared to zero glucose ($p < .025, < 0.005, < 0.005$ respectively). In parallel to this experiment the level of fructose 2, 6-bisphosphate was measured in cardiac myocytes.**Figure 3.25** shows fructose 2,6-bisphosphate level was significantly increased at all glucose concentrations compared to cell incubated with zero glucose.

Previous work with perfused hearts (Clark *et al.;* 2004) showed that palmitate caused a significant increase in AMPK activity. On that basis we reasoned that heart PFK-2 activity might be increased by palmitate. **Figure 3.26** shows that in heart perfused with palmitate under conditions which are known to activate AMPK, there was a slight but non–significant decrease in

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PFK-2 activity. Similary the levels of F2,6BP were not significantly decreased in the presence of

palmitate (**Figure 3.27).**
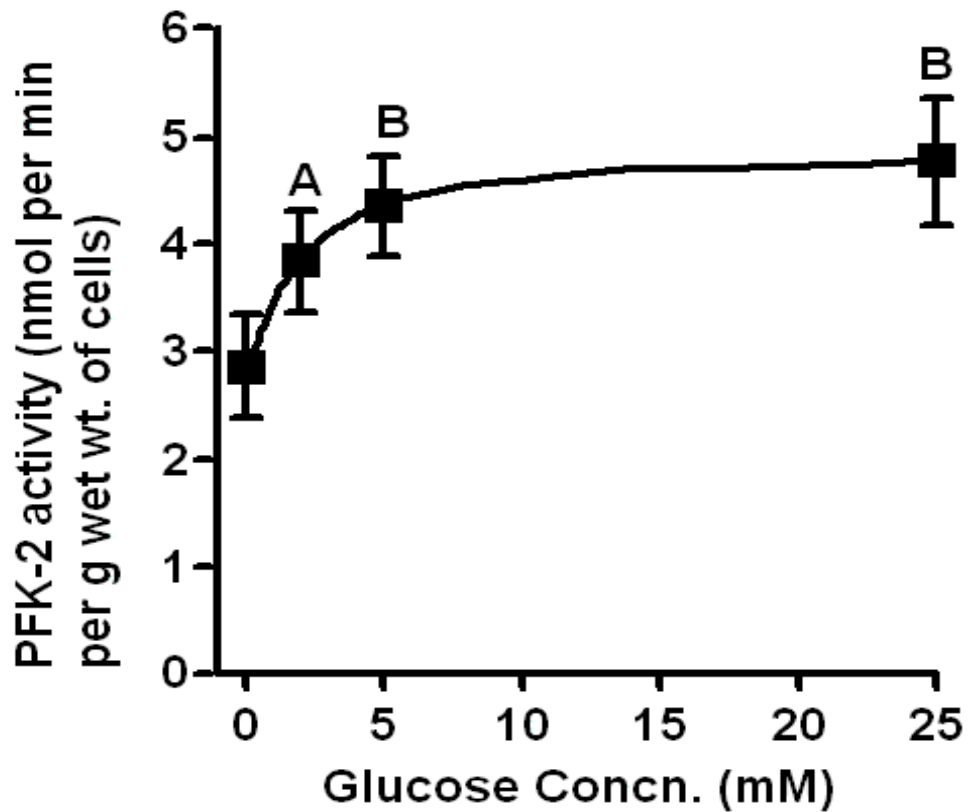
Figure 3.24: **The effect of glucose concentration on myocyte phosphofructokinase -
2 (PFK-2) activity**. Myocytes were incubated for 1 hour with the indicated concentrations of
glucose followed by freeze-stop and assay of PFK-2 activity. The values are means ± S.E.M.
from 9 independent myocyte preparations. A, B indicate $P<0.025$, <0.005 respectively versus
zero glucose (paired tests).
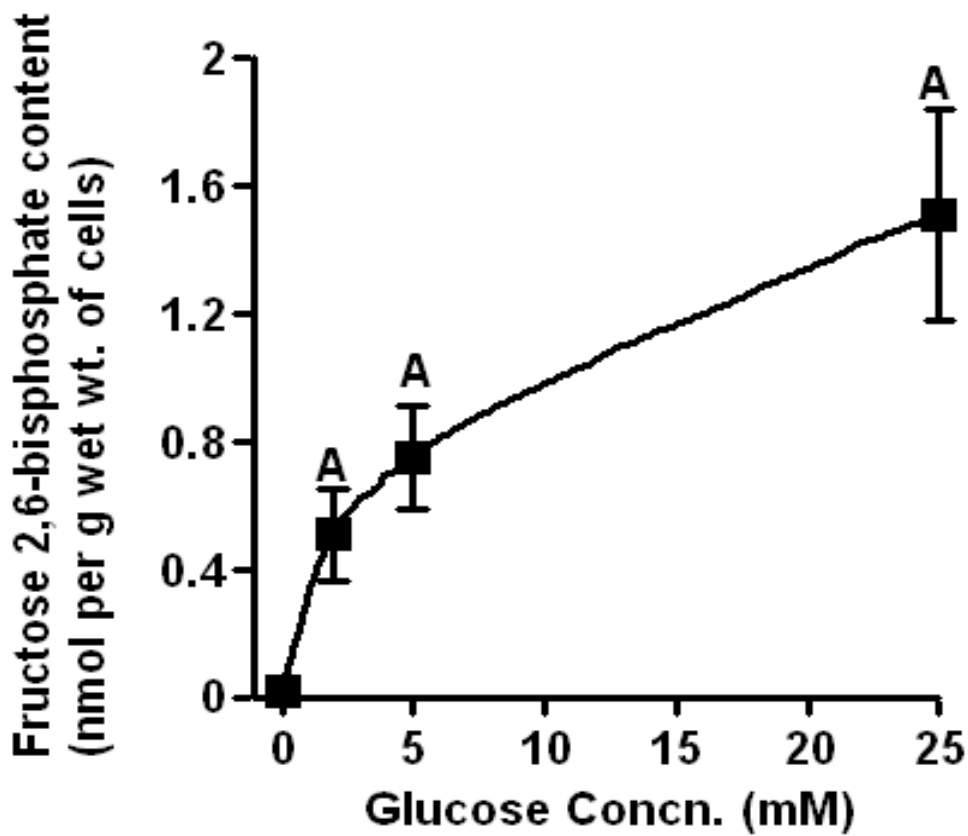
Figure 3.25: **The effect of glucose concentration on the myocyte content of fructose 2,6-bisphosphate**. Myocytes were incubated for 1 hours with the indicated concentrations of glucose followed by freeze-stop and the measurement of fructose 2,6-bisphosphate content. The values are means ± S.E.M. from 5 independent myocyte preparations. A indicates P<0.0005 versus zero glucose (paired tests).
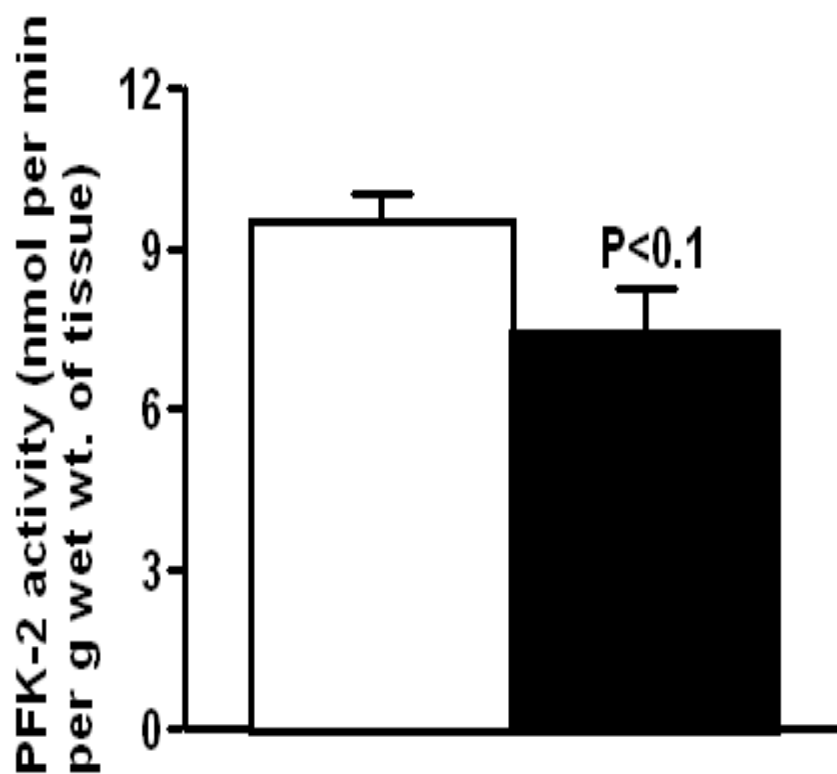
Figure 3.26: **Phosphofructokinase-2 (PFK-2) activities in perfused rat hearts.**
Hearts were perfused for 1 hours with 5 mM glucose without (open symbol) or with 0.5 mM sodium palmitate (filled symbol) followed by freeze-stop and assay of PFK-2 activity. The values are means ± S.E.M. from 9 hearts. As indicated, palmitate did not significantly affect PFK-2 activity (unpaired test).
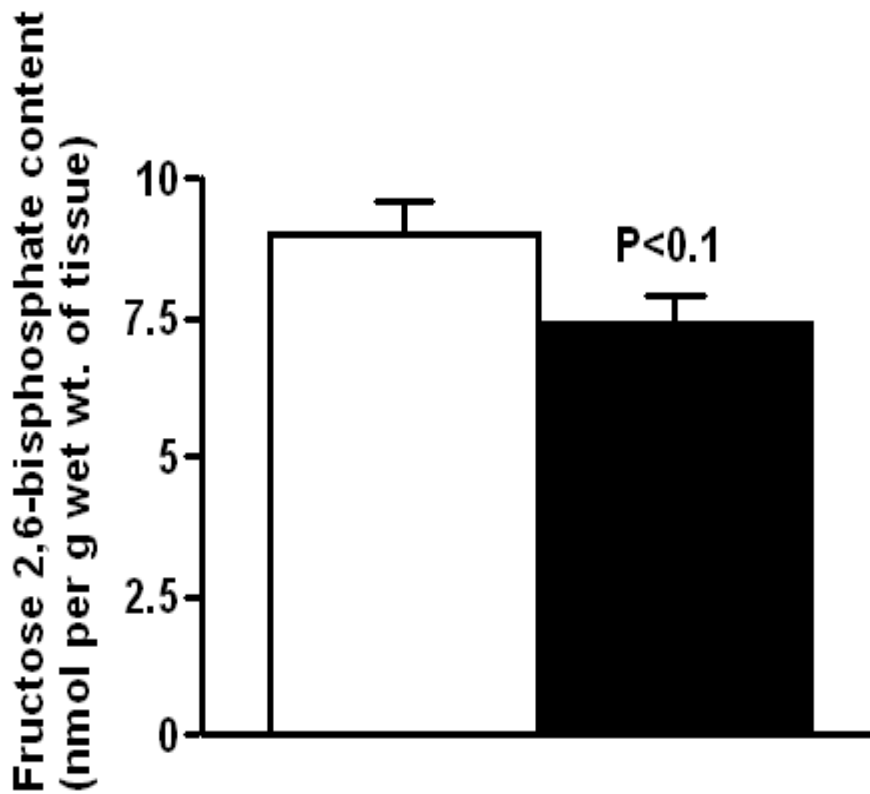
Figure 3.27: **Fructose 2,6-bisphosphate content of perfused rat hearts**

Hearts were perfused for 1 hours with 5 mM glucose without (open symbol) or with 0.5 mM sodium palmitate (filled symbol) followed by freeze-stop and assay of fructose 2,6-bisphosphate. The values are means ± S.E.M. from 5 hearts. As indicated, palmitate did not significantly affect fructose 2,6-bisphosphate content (unpaired test).

To investigate the effect of palmitate on Fru 2,6bisphosphate level in cardiac myocytes, first AMPK activity was investigated under this condition. **Figure 3.28** shows that palmitate significantly increased AMPK activity compared to the control. To examine whether the increased on AMPK activity by palmitate had an effect on the level of fructose 2,6-bisphosphate level in cardiac myocyte, fructose 2,6-bisphosphate level was examined under similar conditions.

Figure 3.29 shows that the levels of F 2,6BP at 15, 30 or 60 minutes were significantly decreased in cells incubated with palmitate compared to the control. This result suggests that in the presence of fatty acid the level of allosteric inhibitors of FPK-2 might increase, and these could have negated any activating effect of AMPK.

Hue and co-workers have observed in isolated working rat heart that glycolysis was stimulated to different extents by increasing the concentration of glucose, by increasing the workload or addition of insulin (Hue and Rousseau, 1993). Under all the condition tested, an increase in fructose 2, 6-bisphosphate content was observed. Similar results were observed in hepatic cells when liver was perfused with 40 mM glucose. The mechanism of the stimulation of hepatic glycolysis by glucose is unclear (Kabashima et *al,;* 2002). In this circumstance, elevated glucose levels might cause a covalent modification of PFK-2/FBPase-2, by a mechanism which overrides any effect resulting from glucose-induced suppression of AMPK, thus leading to the activation of glycolysis. There is substantial evidence to suggest that glucose increases the fructose 2, 6-bisphosphate level. The most convincing lines of evidence come from a demonstration carried out in pancreatic and MIN6 cell line. Glucose may have an impact on the fructose 2, 6- bisphosphate content by covalent modification of PFK-2/FBPase or through changes in metabolite concentration that act as activators or inhibitors of either the kinase or bisphosphatase (Catherine *et al.;* 2008).
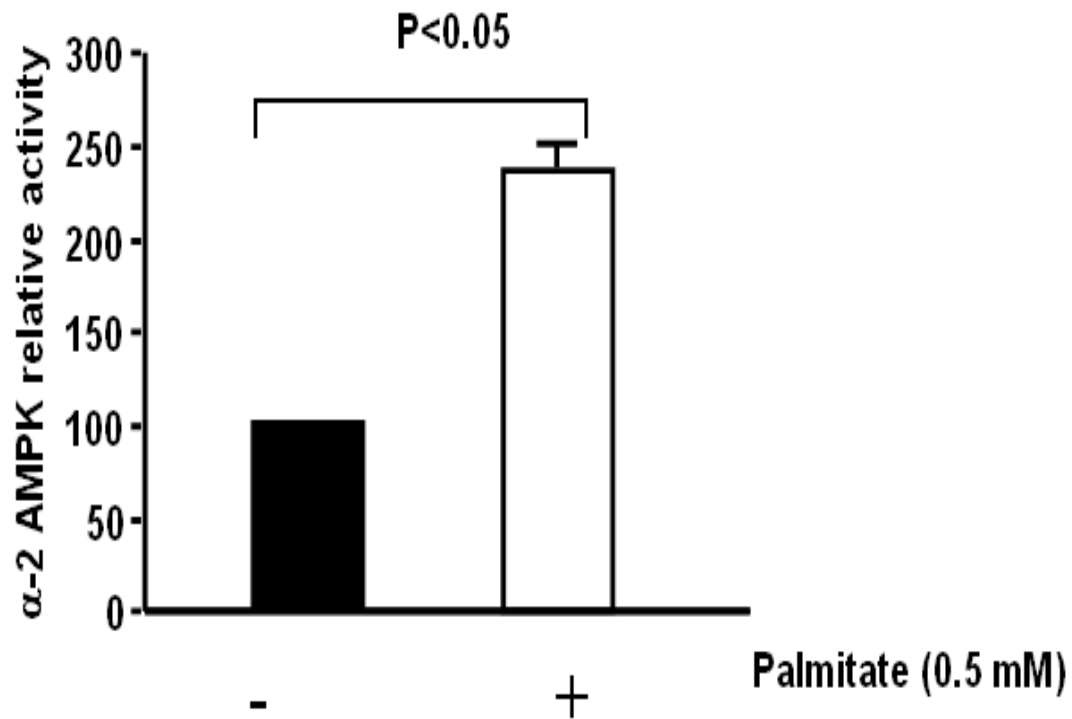
Figure 3.28: **The effects of palmitate on myocyte α-2 AMPK activity.**

Myocytes were incubated for 1 hour with 5 mM glucose without or with 0.5 mM palmitate as indicated. The values are means $\pm$ S.E.M. expressed as percentages of the measurements with 5 mM glucose alone and are from 5 independent myocyte preparations $P<0.05$, Paired test.
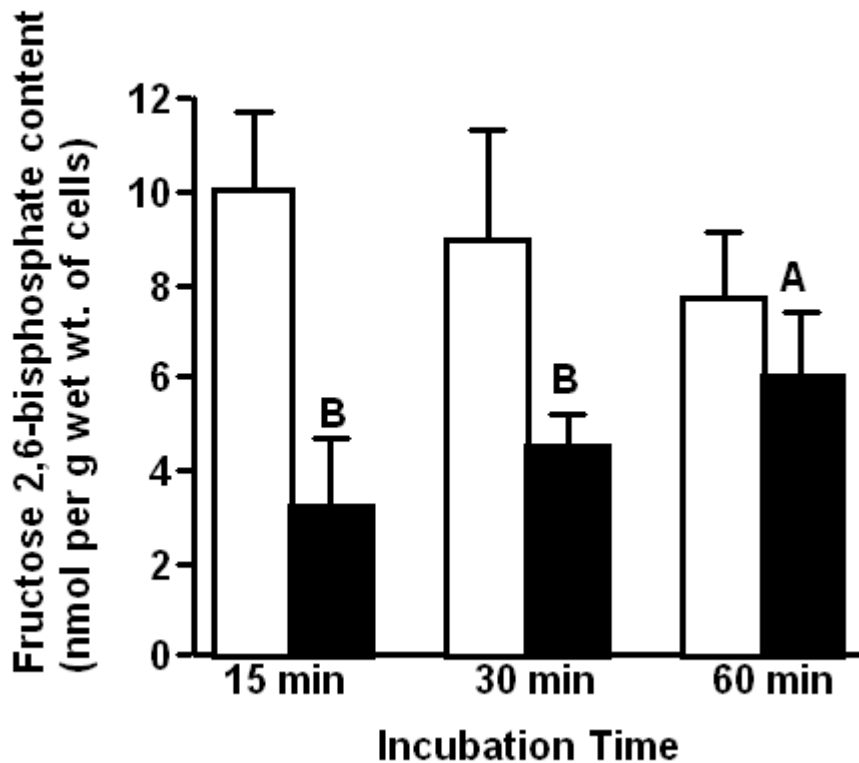
Figure 3.29: **The effect of palmitate on the myocyte content of fructose 2,6-bisphosphate.** Myocytes were incubated for the indicated times with 5 mM glucose without (open symbols) or with 0.5 mM sodium palmitate (filled symbols) followed by freeze-stop and assay of fructose 2,6-bisphosphate. The values are means $\pm$ S.E.M. from 7 independent myocyte preparations.

A, B, indicate $P<0.025$, <0.005 respectively for effects of palmitate (paired tests

3.3.3 eNOS phosphorylation

A potential downstream target of AMPK signalling is the nitric oxide pathway. Ischemic activation of AMPK is accompanied by an increase in eNOS phosphorylation and activation in the heart rate (Chen *et al.;* 2000). Nitric oxide (NO) can enhance ADP-ribosylation of a 37-KDa cytosolic protein, later identified as GAPDH (Dimmeler & Brune, 1992), a key enzyme involved in the glycolytic pathway. A similar action of NO has been documented in neuronal cells (Zhang & Snyder, 1992). Other researchers found that as eNOS increases glucose transport there is an indirect stimulatory action of NO on PFK-2 in neurons (Almeida *et al.;* 2004). Although the mechanisms are still poorly defined, convincing evidence has been provided that eNOS plays an important role in the regulation of myocardial substrate metabolism (Depre C *et al.;* 1989). Previous studies have outlined the involvement of eNOS in glucose metabolism by a stimulation of glucose uptake (Young *et al.;* 2004) mediated by AMPK. In this study cardiac myocyte eNOS Ser 1177 phosphorylation was investigated under different conditions.

Figure 3.30 shows that no effect of glucose concentration, insulin or AICAR on eNOS Ser1177 phosphorylation could be detected. Unfortuntely eNOS appeared to be expressed at low levels in the myocytes and this low level of expression may have precluded the detection of effects**.** This result is in agreement with the results of other groups (Schulz, *et al.;* 2004)
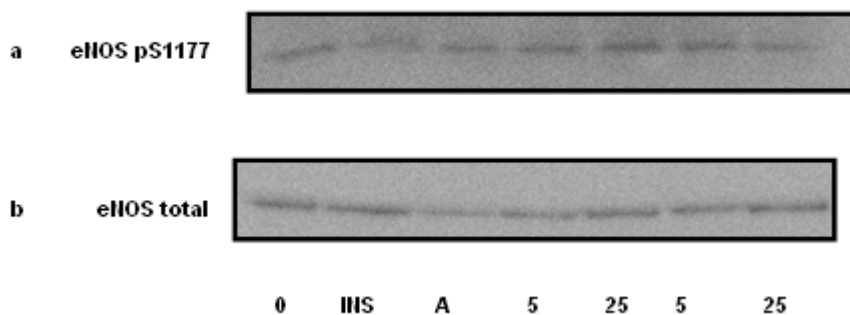
Figure 3.30: **Effect of glucose, insulin and AICAR on eNOS Ser1177 phosphorylation.** Phosphorylation of eNOS Ser1177 was examined after incubation of cardiac myocytes for 1 hour with 10 nM insulin, (INS) 1 mM AICAR (A), 0, 5 , or 25 mM glucose. Cell lysates were analysed by western blotting using (a) anti-phospho-e –NOS antibody and (b) an antibody against total e-NOS. The results are from 3 independent myocyte preparations. The above is representative blot.

3.3.4 Conclusion

In this study, ACC227 phosphorylation was decreased as glucose concentration was increased. The extended N-termial sequence of ACCβ has been proposed to regulate the association of the enzyme with the mitochondrial outer membrane (He *et al.;* 1996). A later study has shown ACCβ to be localised on the mitochondrial outer membrane in avariety of cell type (Abu- Eliheiga *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 *et al.;* 1993). This activation is not due to a change 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 ACCβ may regulate this association. The activity of ACC may 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 (Saha *et al.;* 2002). Purified rat ACCβ is phosphorylated and inactivated in *vitro* by PKA and AMPK (Dyck *et al.;* 1999)

PFK-2

Cardiac ischemia or anoxia increase glycolysis by activation of PFK-2 and increases glucose transport. This effect is mediated by AMPK. AMPK phosphorylates heart PFK-2 on Ser 466 which leads to its activation. Therefore, heart PFK-2 is a novel substrate of AMPK and its activation is involved in the Pasteur effect (Marsin *et al.;* 2000). Hue and co-workers showed that activation of PFK-2 did not require PKB, but was mediated by another protein kinase located downstream of PDK1, this kinase was termed WISK (Hue *et al.;* 2000). Likewise the effect of glucose to activate PFK-2 cannnot require PKB because I have showed that PKB phosphorylation is unaffected by varying glucose concentration (**Figure 3.14).** Since the activation of PFK-2 activity in response to increasing glucose persisted through freeze-stop and lysis of the cell it seems reasonable to believe that this is due to some covalent modification of PFK-2 (an allosteric effect should not survive this treatment). However at this stage we cannot differentiate between the possibility that glucose alter the activity of a protein kinase which modifies PFK-2 or that

glucose is altering of a protein phosphatase for which PFK-2 is a target. At present there is little kown about the protein phosphatases which dephosphorylate heart PFK-2 (personal communication from Rider to Saggerson). The protein kinases which can phosphorylate heart PFK-2 have been reviewed by Rider *et al.;* 2004 and include the conventional Protein kinase C, WISK, AMPK, Ca CAMK, protein kinase, p70S6 kinase and p90 rsk. AMPK, protein kinase A and PKB can be ruled out as possible mediators of the glucose effect on PFK-2. Further work is needed to define which kinase or phosphotase mediates the effect of glucose.

eNOS

During myocardial ischemia, the phosphorylation of eNOS at serine residue 1177 close to the carboxyl-terminal is a critical requirement for eNOS activation and has been reported to be mediated by AMPK. (Chen   *et al*.; 1999). The activity of eNOS is also influenced by phosphorylation at threonine residue (eNOS Thr495). Phosphorylation at this site inhibits NO synthesis, whereas dephosphorylation can promote NO synthesis (Mount *et al*.; 2007). In mice metformin significantly increased the phosphorylation of eNOS Ser1177 phosphorylation over the basal level for a period of 24 h. Metformin did not alter eNOS Thr495 phosphorylation at any investigated time point. It has been reported that Metformin also failed to increase NOS Ser117 phosphorylation when administered to AMPKα2 dn Tg mice. Similarly, eNOS Thr495 phosphorylation remained unchanged in AMPKα2 dn Tg mice administered metformin.(Andrina *et al*.; 2008). From the above studies eNOS phosphorylation is more detectable after at least two hours. In the present study the cells were incubated only for 1 hour. This might be the reason why changes in phosphorylation were undetecable even in the presence of AICAR. eNOS is relatively poorly expressed in cardiac myocytes, and this might affect ability to detected change in its phosphorylation.

Section: 4

3.4 Is the effect of variation of glucose concentration to decrease AMPK activity mediated by a product of glucose metabolism?

3.4.1 The effect of glucose on myocyte glycogen content

AMPK β subunits contain a conserved domain that causes association with glycogen. Although glycogen availability is known to affect AMPK regulation in *vivo*, the molecular mechanism for this has not been clear. It has been reported that AMPK is inhibited by glycogen, particularly preparations with a high branching content. Oligosaccharide with single α1-6 branch is an allosteric inhibitor that also inhibits AMPK phosphorylation by upstream kinases. Removal of the outer chains of glycogen using phosphorylase, thus exposing the outer branches, renders inhibition of AMPK more potent. Inhibition by all carbohydrates tested was dependent on the glycogen-binding domain and was abolished by mutation of residues required for carbohydrate binding (Hardie *et al*.; 2008).

Myocardial glycogen content reflects the balance between glycogen synthase and glycogen phosphorylase fluxes. Both of these enzymes are controlled in a complex manner via phosphorylation and key allosteric mediators, including AMP, ATP, inorganic phosphate and glucose-6-phosphate (Neely *et al* .; 1974; Saha *et al.;* 2002). In previous studies using heart tissue extracts, AICAR had no effect on glycogen synthase and glycogen phosphorylase. Other investigators using skeletal muscle have found contrary evidence, that AICAR activates glycogen phosphorylase and stimulates glycogenolysis (Young *et al.;* 1996). In this study to test whether the increase in stored glycogen is associated with decreased AMPK activity, cells were incubated in 0, 5, and 25 mM glucose for an hour then treated as described in **section 2.9.1** and the extracted glycogen was hydrolysed and assayed by the method described in **section 2.9.1**. **Figure 3.31** shows that the glycogen content in cells was not significantly altered within the glucose concentration range of 5 to 25 mM within which AMPK activity is normally halved.

Hence the decrease in AMPK activity on going from normal glycemic conditions (5mM) to severe hyperglycemia (25mM) cannot be attributed a to a change in glycogen content. The essentially zero glycogen content in cells incubated with zero glucose shows how starved those cells are this complements the significant decrease in total adenine nucleotides under this condition. (**Table3.1**)
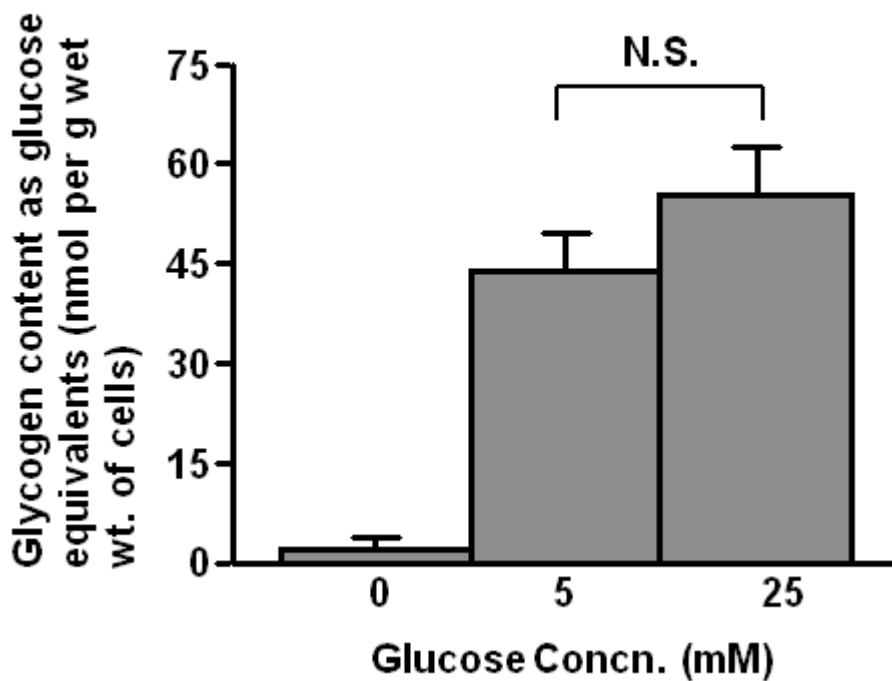
Figure 3.31: **The effect of glucose concentration on myocyte glycogen content**. Myocytes were incubated with the indicated concentrations of glucose for 1 hour followed by extraction and measurement of the glycogen content. The values are means ± S.E.M. from 6 independent myocyte preparations. N .S. indicates that there was no significant difference between the glycogen contents at 5 and 25 mM glucose (P<0.15, paired test).

3.4.2 The effect of glucose on AMPK activity may be one of mediated by its metabolites

Glucose metabolism occurs in three stages - glucose uptake, glycolysis and glucose oxidation. Glucose uptake in the heart occurs via facilitated transport by the glucose transporters, Glut-1 and Glut-4 (Brownsey *et al.,* 1997). The inhibitory effect of glucose on AMPK activity could be due to either a direct action of the sugar itself or due to one of the products generated by its metabolism. To distinguish between direct and an indirect effect of glucose, the effect of a non-metabolizable analogue of glucose on AMPK activity was examined.**Figure 3.32** shows glucose enter the cell through its transporters in the heart GLUT1 and GLUT4. Glucose in cardiac myocyte is phosphorylated by hexokinase to glucose 6-phosphate which is metabolised further**. Figure 3.33** shows that the glucose analogue, 3-O-methy-D-glucose failed to decrease AMPK activity below the level seen with zero glucose. Therefore it seemed more likely that a glucose metabolite might be responsible of the effect of glucose on AMPK activity. The next approache was to try to establish which metabolite or pathway might be responsible for this effect.

3.4.3 Glycolysis

Upon entering the cell, glucose can be metabolised along three different pathways, glycolysis, the pentose phosphate pathway and the hexosamine biosynthetic pathway as it **is** shown in **Figure 3.34**. This prompted us to examine which pathway might be responsible for decreasing AMPK activity. Therefore some metabolites of the glycolytic pathway were tested. Firstly cardiac myocytes were exposed to 2-deoxyglucose (2-DOG). Besides possibly mimicking an effect of free glucose, 2-DOG can be phosphorylated by hexokinase to yield 2-DOG 6-phosphate which is not metabolized further in glycolysis but which might possible mimic a regulatory effect of glucose 6-phosphate. However like 3-O-methyglucose, 2-DOG was failed to decrease AMPK activity below the level seen with zero glucose (**Figure 3.33).** As a second approach cardiac

myocytes were incubated with the glycolytic products lactate and pyruvate as the indicated concentrations.

 Figure 3.35 shows a significant increased in AMPK activity in cells incubated in the presence of 3 mM pyruvate when compared with the control cells ($p < 0.005$). However pyruvate did not mimic the effect of glucose to decrease AMPK activity. The effect of pyruvate on AMPK activity is unexpected. It should be noted that the concentration of pyruvate which was used in this experiment was supra-physiological.

In order to determine whether lactate could affect AMPK activity in cardiac myocytes, cells were incubated with various lactate concentrations and α-2 AMPK activity was measured. Figure **3.36** shows that there was no significant difference in AMPK activity in cells incubated with lactate concentrations in the range of 0.5 - 2 mM, in comparison to the control cells. However, the activity of AMPK was significantly increased at the slightly supra-physiological concentration of 5 mM lactate in comparison to the control ($p < 0.005$).

During metabolic stress – such as ischemia – the heart quickly adapts to the restricted oxygen and nutrient supply, and undergoes a dramatic switch from aerobic to anaerobic metabolism. Pyruvate produced from glycolysis is converted to lactate. There is a growing body of evidence which suggests that AMPK is considered to be an important mediator in the control of glucose and fatty acid metabolism during metabolic stress. From the above results, one could conclude that AMPK activation occurs at high lactate concentrations which could act in parallel to the ischemic condition. Additionaly,   these findings suggest that pyruvate and lactate are not the candidates as mediators of the effect of glucose to decrease AMPK activity.
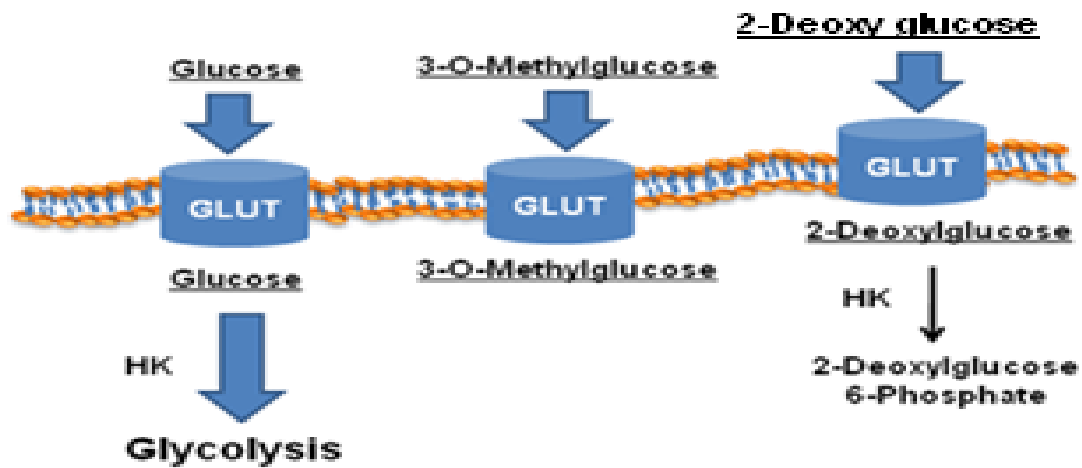
Figure 3.32 shows 3-O-methylglucose is not phosphorylated by hexokinase whereas 2-deoxyglucose is phosphorylated to 2-deoxyglucose 6-phosphate. Neither sugar is further metabolized.
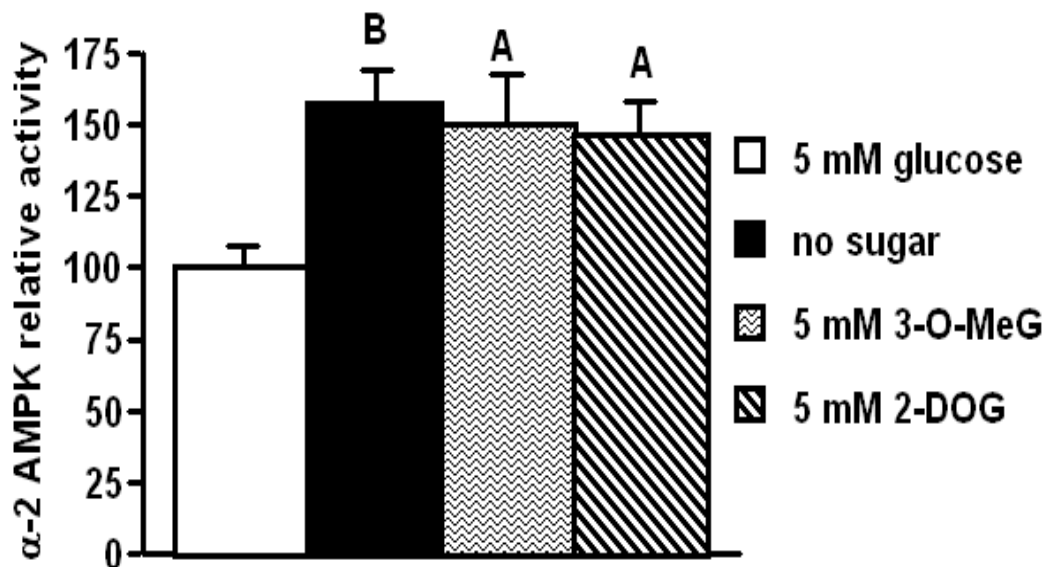
Figure 3.33: 3-O-methylglucose (3-O-MeG) and 2-deoxyglucose (2-DOG) did not mimic the effect of glucose to decrease myocyte α-2 AMPK activity.

Myocytes were incubated for 1 hour with the indicated additions. The values are means ± S.E.M. expressed as percentages of the paired 5 mM glucose values. The numbers of independent myocyte preparations were: 5 mM glucose, 12; with no sugar, 5; with 5 mM 3-O-MeG, 5; with 5 mM 2-DOG, 7.A, B, indicate $P<0.01$, <0.005 respectively versus the 5 mM glucose value (paired tests).
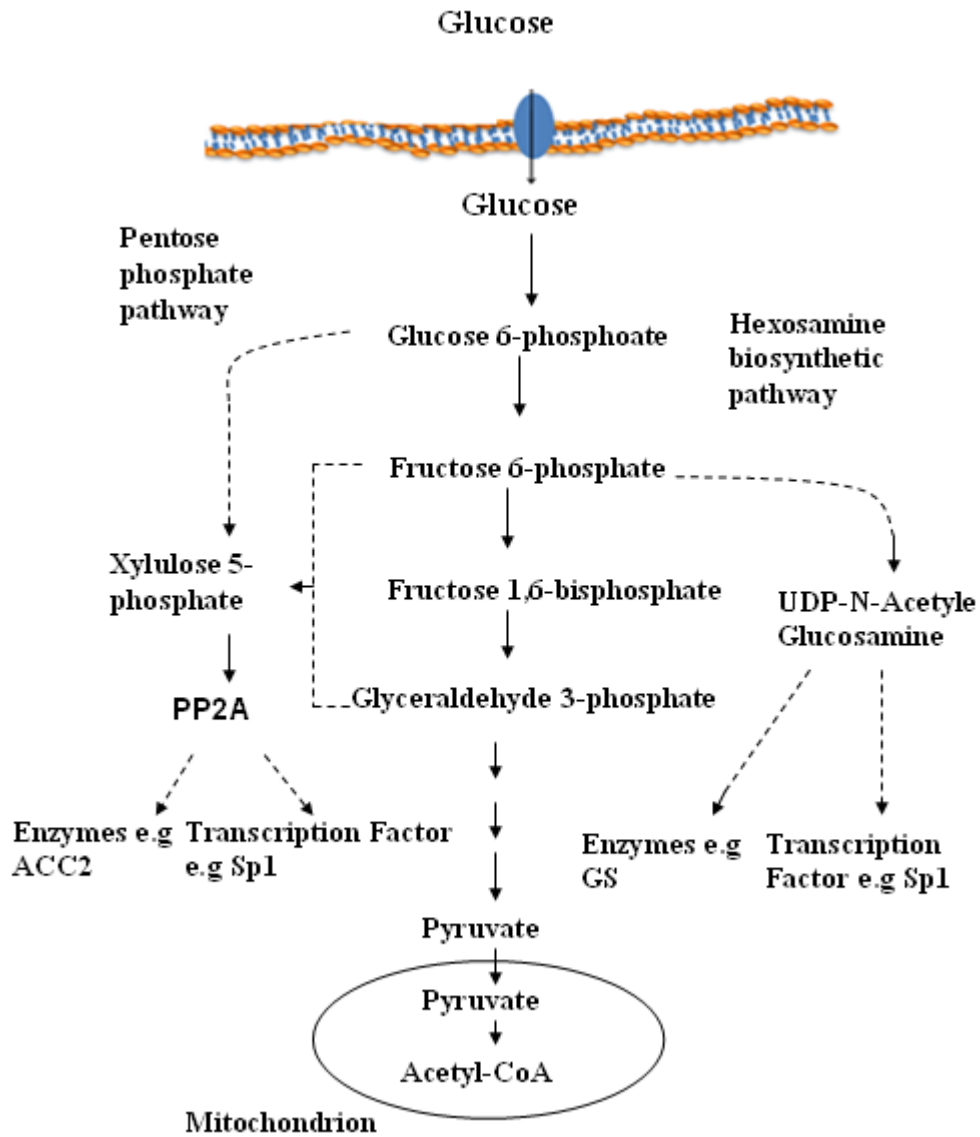
Figure 3.34 Hypothetical mechanism of glucose sensing in cardiac myocyte in response to an alteration in glucose level. Accumlation of glycolytic intermediates result in an increased flux through the pentose phosphate pathway via glucose 6-phosphate dehydrogenase and the hexosamine biosynthethic pathway via glutamine fructose-6-phosphate amidotransferase, resulting in accumulation in xylulose 5-phosphate and UDP N-acetyl glucosamine, respectively. These metabolites affect the activity of various enzymes and transcription factors for phosphorylation and/or glycosylation events.
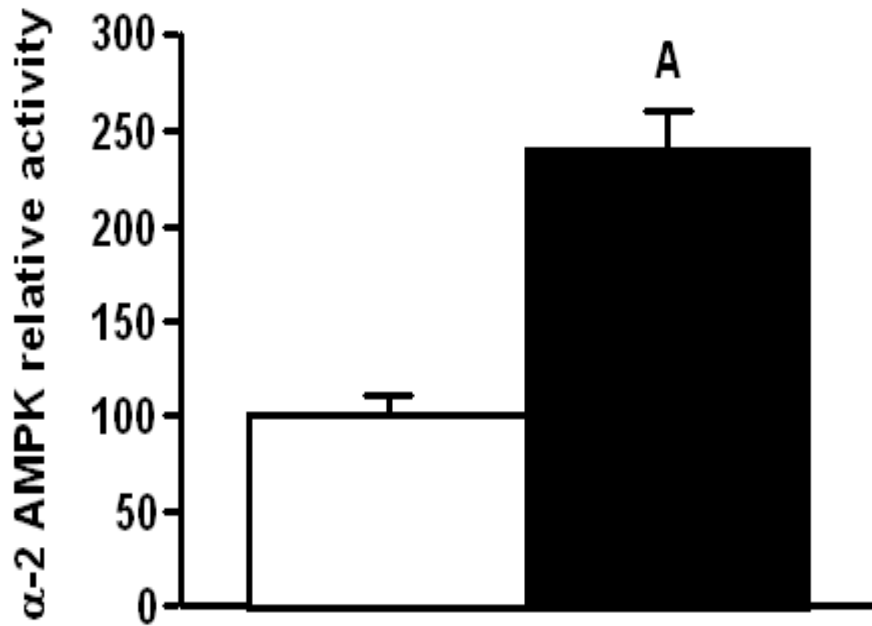
Figure 3.35: the effect of pyruvate on myocyte α-2 AMPK activity.

Myocytes were incubated for 1 hour with 5 mM glucose without (open symbol) or with 3 mM sodium pyruvate (filled symbol). The values are means ± S.E.M. expressed as percentages of the zero pyruvate measurements and are from 8 independent myocyte preparations.A indicates $P<0.0005$ for the effect of pyruvate (paired test).
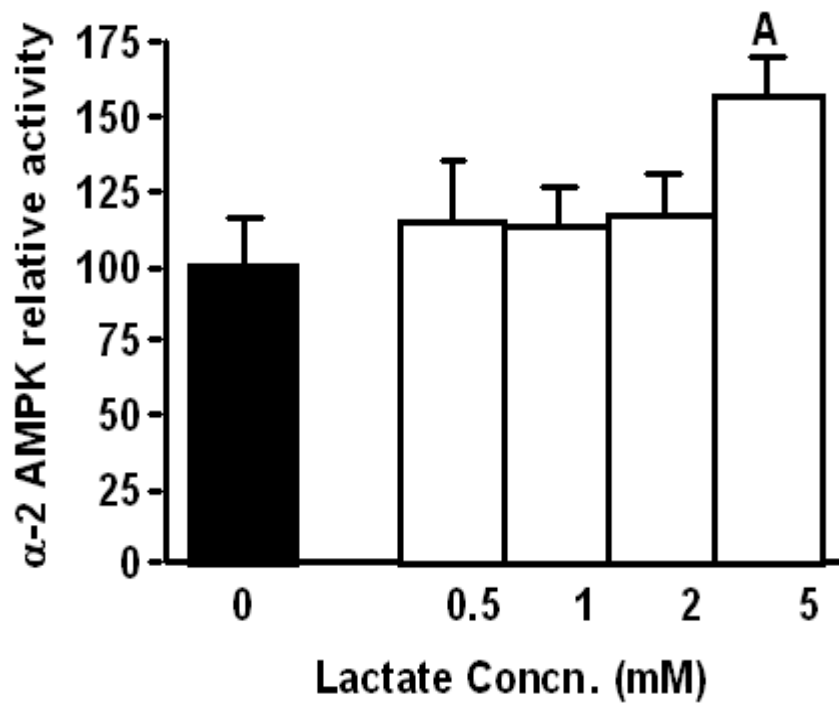
Figure 3.36: **The effect of L-lactate on myocyte α-2 AMPK activity.**

Myocytes were incubated for 1 hour with 5 mM glucose alone (filled symbol) or with the indicated concentrations of sodium L-lactate (open symbols). The values are means ± S.E.M. expressed as percentages of the zero lactate measurements and are from 10 independent myocyte preparations. A indicates $P < 0.005$ for the effect of 5 mM lactate (paired test).

3.4.4 The hexosamine biosynthetic pathway

Once glucose is phosphorylated to glucose 6-phosphate, it is converted to fructose 6-phosphate in the next step of glycolysis. Fructose 6-phosphate can be converted into UDP-N-acetylglucosamine (UDPGlcNac) by glutamine fructose 6-phosphate-amidotransferase (GFAT), the first and rate-limiting enzyme on the hexosamine biosynthetic pathway. UDPGlcNac serves as the substrate for the O-GlcNacylation of protein by the enzyme O-GlcNac transferase (OGT). O-GlcNacylation has been shown to compete for the phosphorylation sites of some signalling proteins and transcription factors leading to modification of activity of these proteins (McClain DA 2002). In order to test the effect of inhibition of GFAT by azaserine on AMPK activity, cardiac myocytes were incubated with 5 mM glucose in the presence or absence of 30 μM azaserine; an inhibitor of GFAT.

 Figure 3.37 shows (a) AMPK activity was not significantly difference in cells incubated in the presence of azaserine compared to the control. (b) Azaserine failed to alter the phosphorylation of AMPK at α-Thr172. Also no change was observed on the total AMPK protein with azaserine. This result suggests that the effect of glucose on AMPK activity was unlikely to be mediated by an intermediate formed post the GFAT reaction of the hexosamine pathway.

(a)
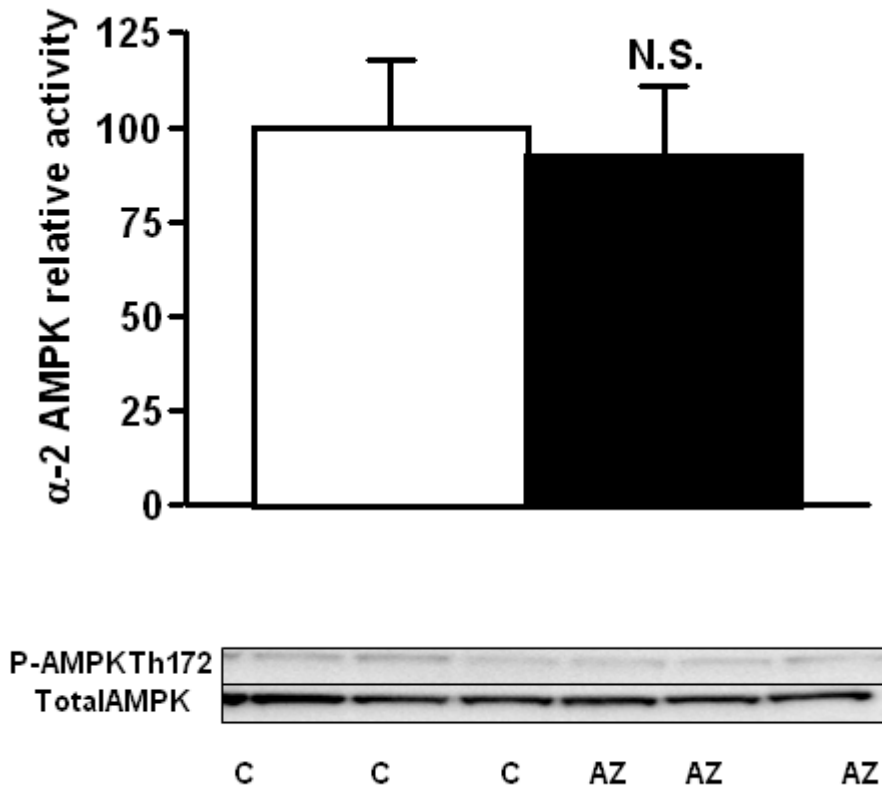
(b)

Figure 3.37: **Azaserine had no effect on myocyte α-2 AMPK activity.**

(a)Myocytes were incubated for 1 hour with 5 mM glucose(C) without (open symbol) or with 30 μM azaserine (filled symbol)(AZ). The values are means ± S.E.M. expressed as percentages of the measurements without azaserine and are from 6 independent myocyte preparations. N.S. indicates that azaserine had no significant effect ($P<0.3$, paired test). (b) The phoshorylation of AMPK α-Thr172 and total AMPK were examined. Cells were lysed and analysed by western blotting using anti-phospho- AMPK α-Thr172 and total AMPK antibody (Representative blot).

3.4.5 The pentose phosphate pathway

Goodwin and co workers (2001), using perfused working rat hearts, showed that flux through the oxidative arm of PPP is very low compared with metabolisation of glucose via glycolytic pathway. However Goodwin *et al.;* 2001 made the conclusion based on the measurement of the detritiation of [5-^{3}H]glucose that there as as much as 20% of the glucose can be taken up by the heart may have some interconversion within non- oxidative arm of the PPP.

To investigate possibility of the involvement of the pentose phosphate pathway metabolites on AMPK activity, cardiac myocytes were incubated in 5 mM glucose in the presence or absence of 0.1and 0.5 μM xylulose or .05 and 0.2 mM ribose or 0.5 and 1 mM xylitol, which is known to be converted to xylulose 5-phosphate within cells.

Figure3.38 shows no significant difference in AMPK activity between the control cells incubated with ribose or xylulose at the treated concentrations. These results suggest that the possibility that the concentrations of these sugars which were used in these experiments were too low. Additionally these sugars might not penetrate into the cells.

Figure 3.39 shows that 1 mM xylitol significantly decreased AMPK activity ($p<0.005$) compared to the control**.** In addition when cells lysate were probed against phospho- AMPK α-Thr 172 and phospho– ACC Ser277 it was difficult to quantify the bands so as to detect the difference between control and treated cells. No change in the AMPK total protein was observed.

 In addition, we examined whether increased glucose could lead to an increase in the level of xylulose-5-phosphate. Xylulose 5-phosphate levels were measured by the method described in **section 2.9.3**. **Figure 3.40** shows that xylulose 5-phosphate levels were significantly increased in cardiac myocytes incubated with 2, 5 and 25mM glucose versus cells incubated with 0 mM glucose ($p< 0.05, < 0.005, < 0.005$ respectively).
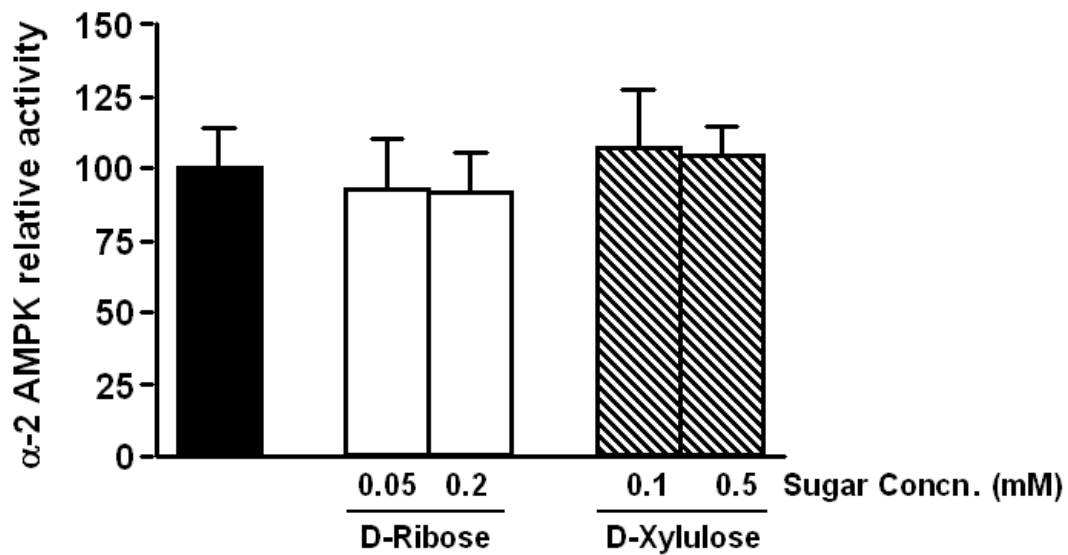
Figure 3.38: **D-ribose and D-xylulose had no effect on myocyte α-2 AMPK activity.** Myocytes were incubated for 1 hour with 5 mM glucose alone without (filled symbol) or with 5 mM glucose together with the indicated concentrations of D-ribose or D-xylulose. The values are means ± S.E.M. expressed as percentages of the measurements with 5 mM glucose alone and are from 5 (ribose) or 4 (xylulose) independent myocyte preparations. No significant effects of ribose or xylulose were observed (paired tests).
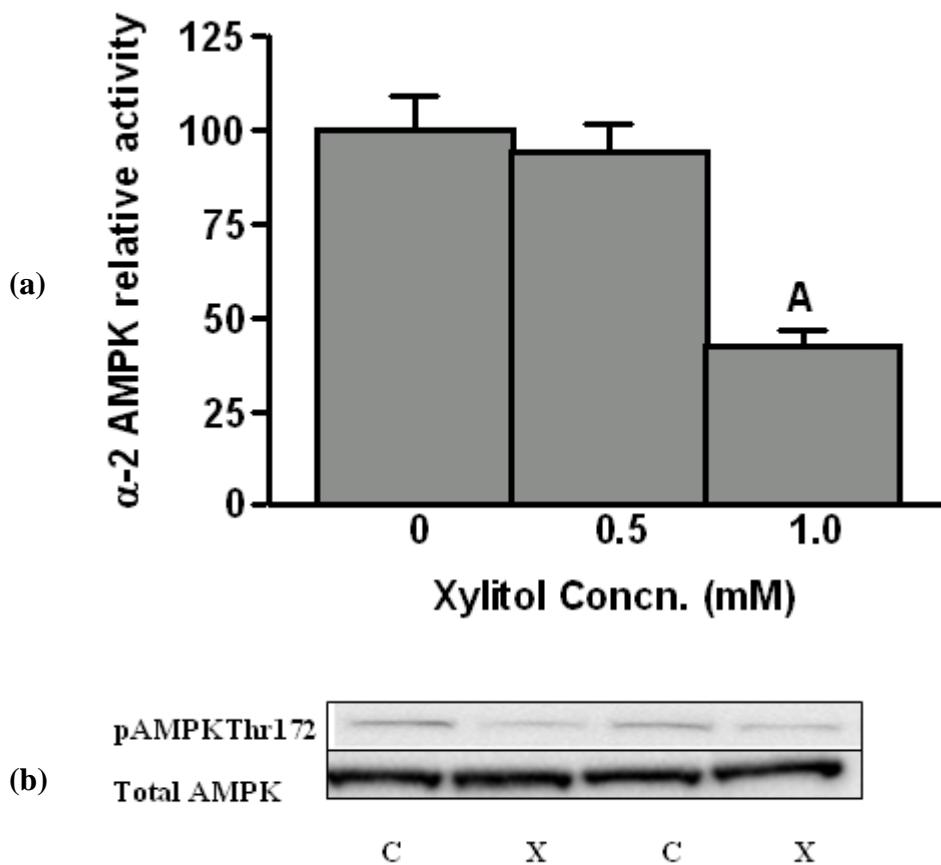
Figure 3.39: The effect of xylitol on myocyte α-2 AMPK activity. (a) Myocytes were incubated for 1 hour with 5 mM glucose and the indicated concentrations of xylitol. The values are means ± S.E.M. expressed as percentages of the zero xylitol measurements and are from 7 independent myocyte preparations. A indicates P<0.0005 compared against zero xylitol (paired test). (b) Representative blot of AMPK phosphorylation at α-Thr172 and AMPK total. The phosphorylation of AMPK α-Thr172 and total AMPK were examined. Cells were lysed and analysed by western blotting using anti-phospho- AMPK α-Thr172 and total AMPK antibody (Representative blot).
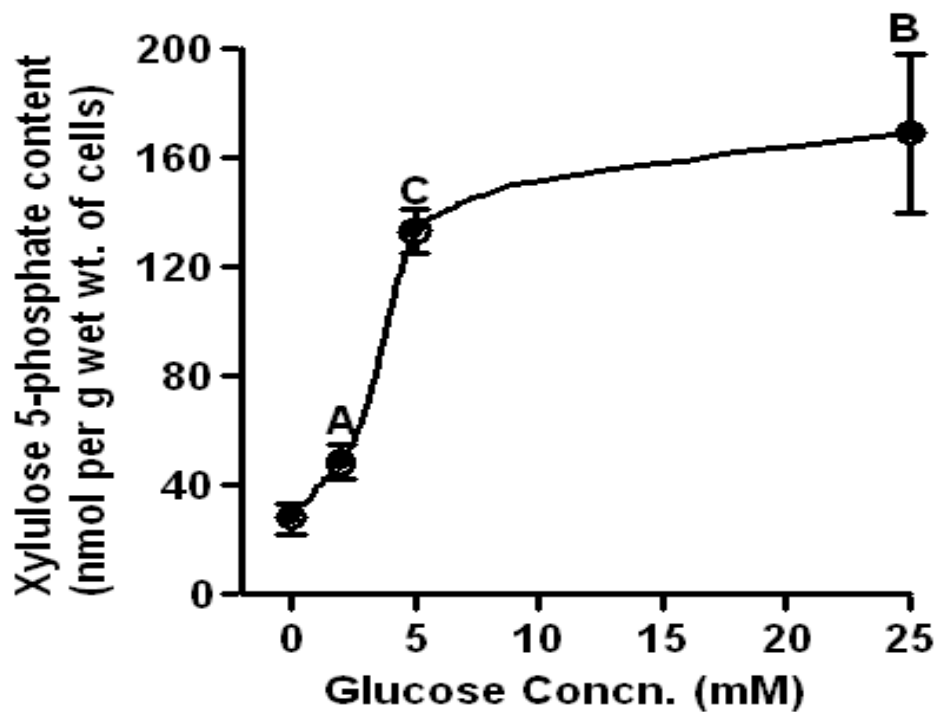
Figure 3.40: **The effect of glucose concentration on the myocyte content of xylulose 5-phosphate**. Myocytes were incubated for 1 hour with the indicated concentrations of glucose followed by freeze-stop and measurement of xylulose 5-phosphate content. The values are means ± S.E.M. from 9 independent myocyte preparations.A, B, C indicate P<0.05, <0.005 <0.0005 versus zero glucose (paired tests).

3.4.5.1 Effect of phenazine methosulphate (PMS) and dehydroepi-androsterone (DHA) on AMPK activity

Two complementary approaches were employed to investigate a possible contribution of the pentose phosphate pathway to AMPK inhibition by glucose in cardiac myocytes. The first approach is the inhibition of glucose-6-phosphate dehydrogenase (G6PD), which functions as the first and rate-limiting enzyme in the pentose phosphate pathway, and is responsible for the generation of NADPH in a reaction coupled with the de novo production of cellular ribose. It has been reported that, in adult cardiac myocytes, G6PD activity rapidly increases in response to cellular oxidative stress, and with the translocation of G6PD to the cell membrane, which can lead to PPP acceleration (Mohit *et al.;* 2003)**.** The inhibition of G6PD results in a decreased PPP rate which can lead to the depletion of reduced glutathione (GSH) levels, and may subsequently cause cardiomyocyte contractile dysfunction.

AMPK activity was examined under conditions when the oxidative arm of the PPP was either inhibited or activated. Firstly cardiac myocytes were incubated in 5 mM glucose in the presence or absence of 30 μM and 60 μM dehydroepi-androsterone (DHA), a non-competitive inhibitor of G6PD, for 1 hour. **Figure 3.41** shows that in cells incubated with of 30 or 60 μM DHA AMPK activity significantly increased by 2- to -3 fold compared with the untreated cells ($p < 0.025$, < 0.005, respectively).

A second approach was to activate this pathway using the artificial electron acceptor phenazine methosulphate (PMS). Cardiac myocytes were incubated in 5mM glucose in the presence of 30 or 50 mM PMS. **Figure 3.42** shows PMS at 30, 50 μM significantly decreased AMPK activity by 28% and 65% respectively compared to the control ($p < .0.005. < 0.0005$ respectively). Furthermore xylulose 5-phosphate was assayed to see the   effect of PMS and DHA on its level. **Figure 3.43** shows that PMS significantly increased xylulose 5-phosphate level compared to the control ($p < 0.005$) whereas DHA significantly decreased xylulose 5- phosphate to a level that was hardly detectable.
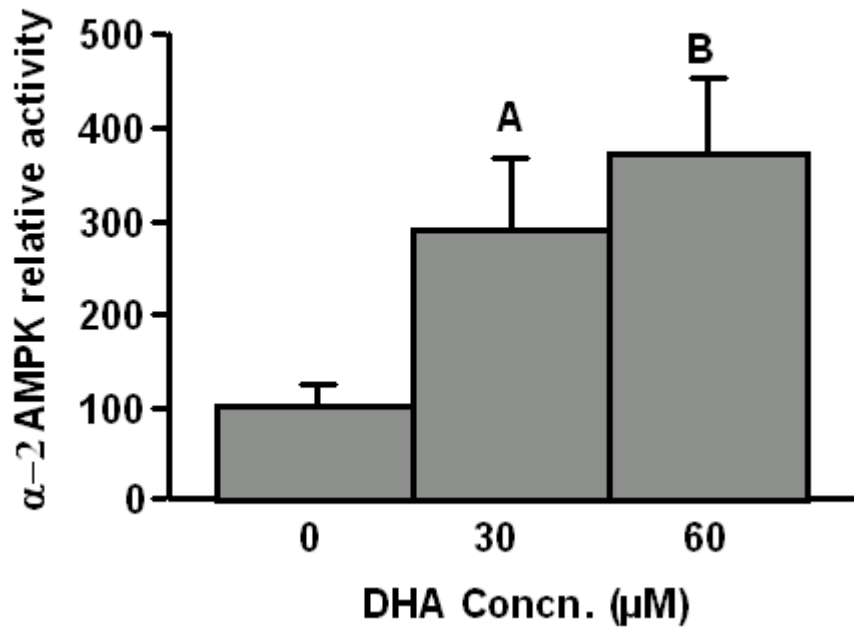
Figure 3.41: The effect of dehydroepiandrosterone (DHA) on myocyte α-2 AMPK activity. Myocytes were incubated for 1 hour with 5 mM glucose and the indicated concentrations of DHA. The values are means ± S.E.M. expressed as percentages of the measurements without DHA and are from 6 independent myocyte preparations. A, B indicate $P < 0.025$, < 0.005 respectively compared against zero DHA (paired tests).
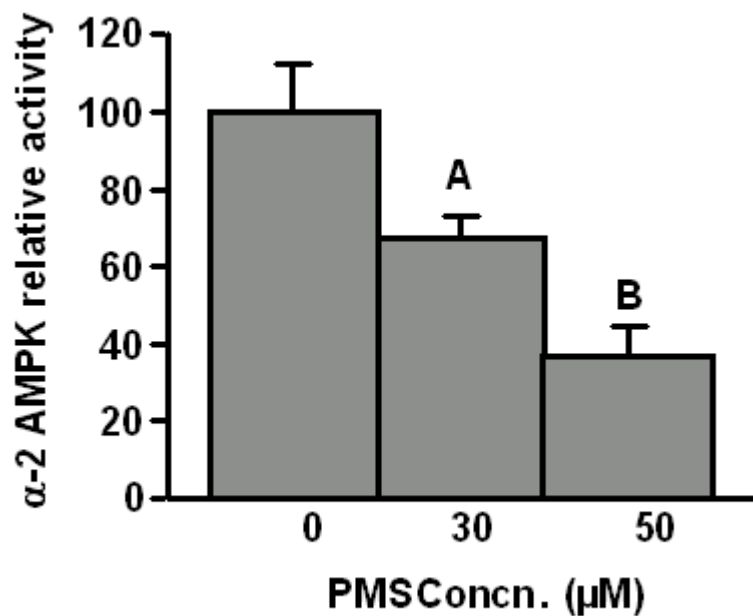
Figure 3.42: **Tthe effect of phenazine methosulphate (PMS) on myocyte α-2 AMPK activity.** Myocytes were incubated for 1 hour with 5 mM glucose and the indicated concentrations of PMS. The values are means ± S.E.M. expressed as percentages of the measurements without PMS and are from 6 independent myocyte preparations. A, B indicate $P<0.005$, $P<0.0005$ respectively compared against zero PMS (paired tests).
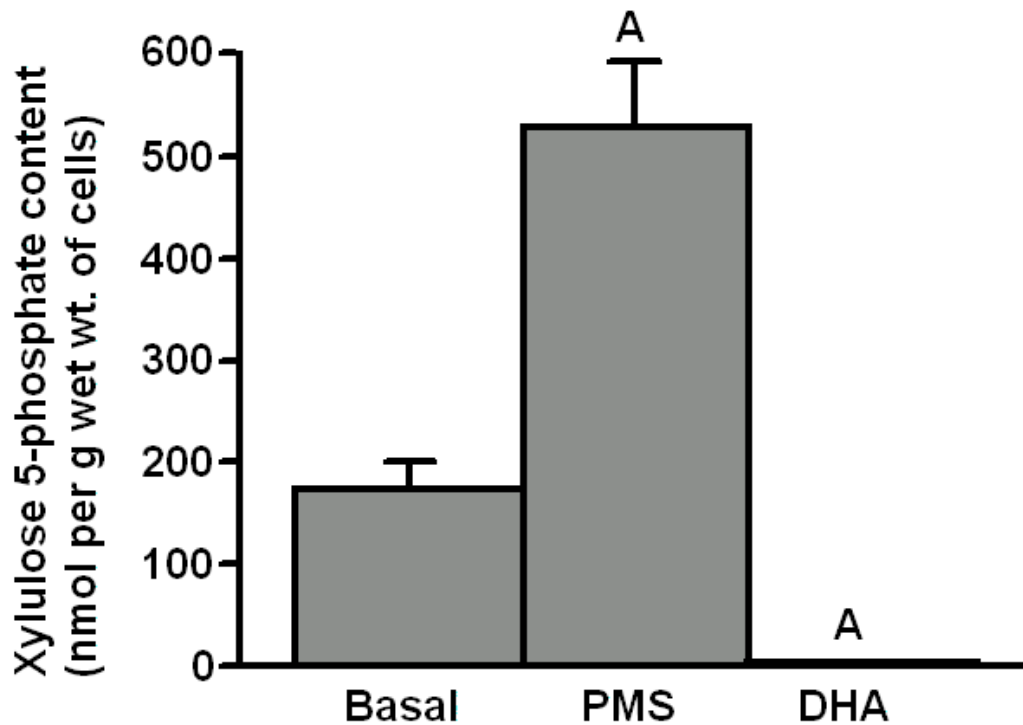
Figure 3.43: **The effects of phenazine methosulphate (PMS) and dehydroepi-androsterone (DHA) on the myocyte content of xylulose 5-phosphate.** Myocytes were incubated for 1 hour with 5 mM glucose alone (basal) or with 5 mM glucose plus either 30 μM PMS or 30 μM DHA followed by freeze-stop and measurement of the xylulose 5-phosphate content. The values are means ± S.E.M. for 8 independent myocyte preparations. In 3 experiments the xylulose 5-phosphate content with DHA was below the limits of the assay and was recorded as zero. The mean content of xylulose 5-phosphate in the presence of DHA was 1.7 ± 0.5 nmol per g wet wt. of cells. A indicates $P<0.0005$ for effects of PMS and DHA (paired differences).

The relationship between α-2 AMPK activity and xylulose 5-phosphate content in myocytes is shown in Figure 3.44. This finding would not be at variance with notion that xylulose 5-phosphate might be part of a process of feedback regulation of AMPK, possibly through activation of PP2A. Further experiments were then conducted with a view to explore a possible link between PPP flux, xylulose 5-phosphate content and myocytes AMPK activity.
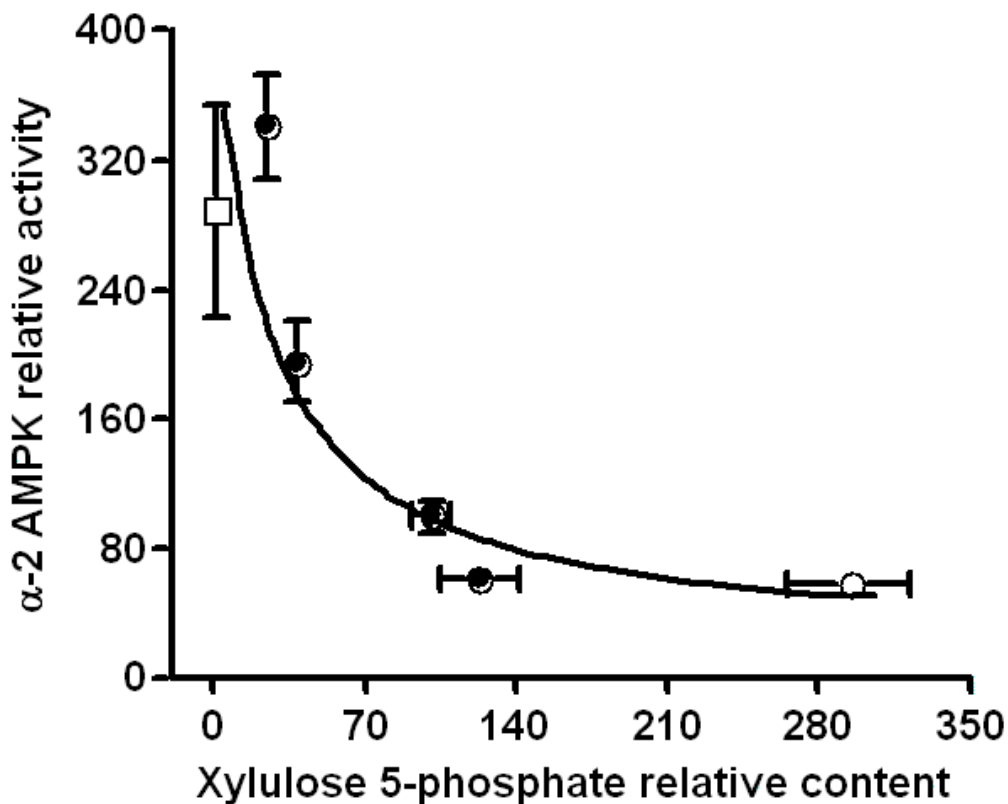
Figure 3.44: **The relationship between the α-2 AMPK activity and the xylulose 5-phosphate content in myocytes.** Values for α2 AMPK activity and for xylulose 5-phosphate content from several different experiments were normalized by expression of these as percentages ± S.E.M. of the measurements made from cells incubated with 5 mM glucose alone where the absolute values were: AMPK activity, 1.71 ± 0.19 pmol per min per mg of 13,000g supernatant protein (n = 56); xylulose 5-phosphate content, 157 ± 14 nmol per g wet wt. of cells (n = 17). Data for AMPK activity were taken from Figures 3.4, 3.11, 3.18, 3.38, 3.33, 3.35**,** 3.39, 3.41, and 3.42. Data for xylulose 5-phosphate were taken from Figures 29 and 39. The minimum number of independent measurements of AMPK activity at any data point was 6 and the minimum number of independent measurements of xylulose 5-phosphate at any data point was 8.Filled circles: varied glucose concentrations between zero and 25 mM. Open square: 5 mM glucose + 30 μM DHA. Open circle: 5 mM glucose + 30 μM PMS. Where S.E.M. bars are not visible they lie within the symbol.

3.4.6 The combined effect of PMS and insulin on AMPK activity

Previous studies have shown that insulin inhibits AMPK activity and α-Thr172 phosphorylation. In the present studies PMS significantly decreased AMPK activity as is shown in **Figure 3.34.** **A**s the result of these two findings, an experiment was performed to see the extent to which PMS and insulin were or were not additive in their effect on AMPK activity. Insulin decreases AMPK activity through PKB whereas PMS might be working through activation of PP2A by Xu5P. **Figure 3.45** shows that there was additively in combined effects of PMS and insulin on AMPK activity at 5 mM glucose. At 25 mM glucose although both PMS and insulin had significant effect on AMPK ativity   but they had no additively of effect in combination. As a speculation 25 mM glucose plus either PMS or insulin gives an approximate rock-bottom AMPK activity which is about 40% of that seen with 5 mM glucose alone. Presumably actions of cellular AMPKKs work to always ensure a certain phosphorylation/activity level of the AMPK.
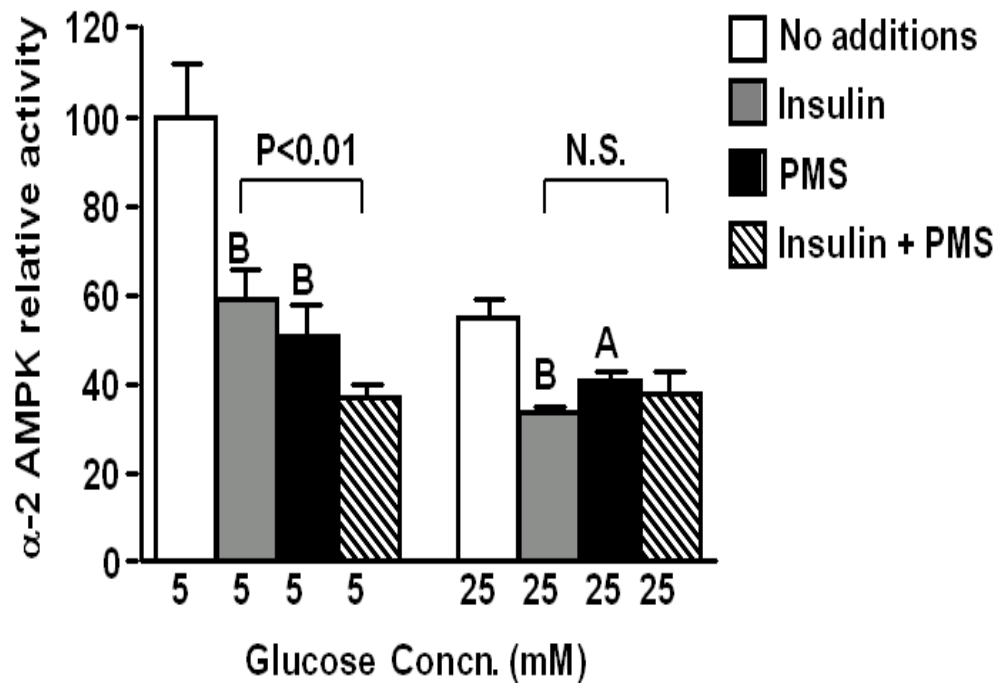
Figure 3.45: **The combined effects of glucose concentration, insulin and phenazine methosulphate (PMS) on myocyte α-2 AMPK activity.** Myocytes were incubated for 1 hour with either 5 mM or 25 mM glucose together with 10 mM insulin or 30 μM PMS as indicated. The values are means ± S.E.M. expressed as percentages of the measurements with 5 mM glucose alone. 8 independent myocyte preparations were used at 5 mM glucose and 5 preparations at 25 mM glucose. A, B indicate $P<0.005$, <0.0005 respectively for effects of insulin or PMS compared with the appropriate basal state (paired tests). The paired test P values shown directly on the figure indicate the significance of the effect of PMS in the presence of insulin. At 5 mM glucose effects of insulin and PMS were partially additive, i.e. PMS decreased AMPK activity by 49% and by 38% in the absence and presence of insulin respectively. Significant additive effects of PMS and insulin were not seen with 25 mM glucose.

3.4.7 Thiamine attenuates the effect of glucose on AMPK

High doses of thiamine might metabolize xylulose 5-phosphate rapidly and therefore not allow it to accumulate to a level suffient to activate PP2A. Cardiac myocytes were incubated with 5 and 25 mM glucose in the presence or absence of 1mM thiamine. **Figure 3.46** shows that when the cells were incubated for 1 hour with 5 mM glucose, thiamine significantly increased AMPK activity by 47% compared to the control (p< 0 .025). In cells incubated for 2 hour in 5 or 25 mM glucose, thiamine significantly increased AMPK activity by 55% and 100% (p< 0.005 <0.005 respectively) compared to the control. The level of phosphorylation of the AMPK at Thr172 and ACC Ser277 were also accessed by Western blotting analysis. **Figure 3.47** shows that thiamine in the presence of 5 mM glucose significantly increased the phosphorylation of AMPK α-Thr172 compared to the control (p<0.05). However, thiamine in the presence of 25 mM glucose also showed increased on the phosphorylation of AMPK αThr172, but this increase was not statistically significant, yet a noticeable increase of phosphorylation of AMPK at 25 mM glucose can be seen on the blot **(Figure 3.47).**

Figure 3.48 shows that thiamine at 5 and 25 mM glucose increased the phosphorylation ACC at Se277 significantly compared to the control (p < 0.025, <0.005 respectively).

These results suggest that thiamine may pay a role in the regulation of AMPK activity and its downstream target ACC. The mechanism by which thiamine increases AMPKactivity is unclear. The possible explanations are (i) that thiamine might decrease xylulose 5-phosphate content and (ii) that thiamine might increase the cellular AMP/ATP ratio,
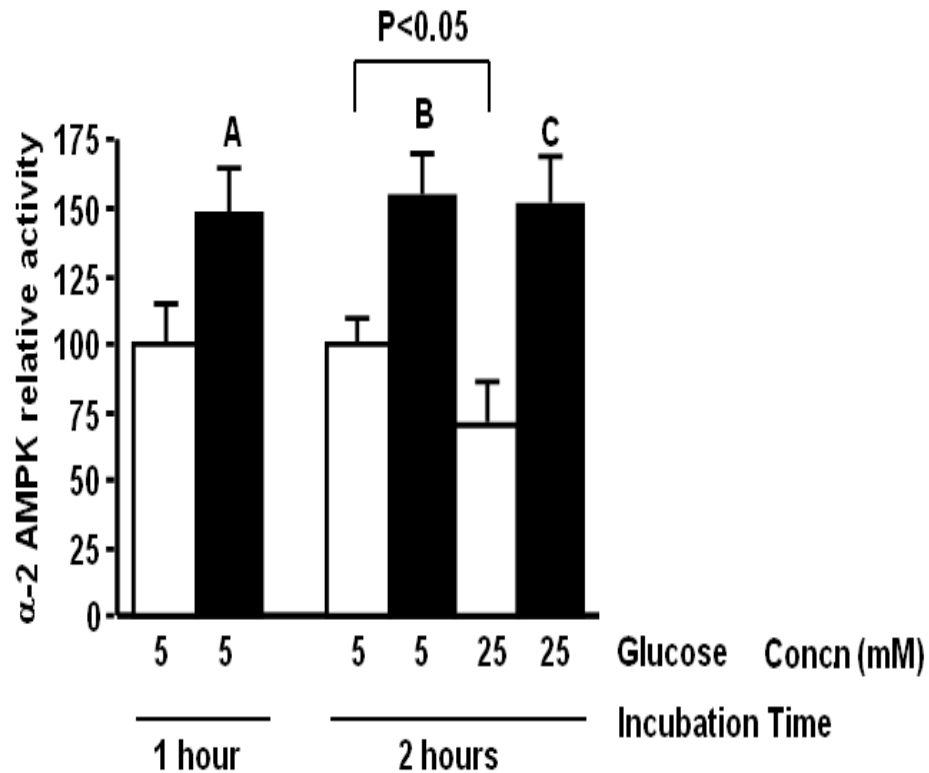
Figure 3.46: The effect of thiamine on myocyte α-2 AMPK activity. Myocytes were incubated for 1 or 2 hours with the indicated concentrations of glucose without (open symbols) or with 1 mM thiamine (filled symbols). The values are means ± S.E.M. expressed as percentages of the 5 mM glucose measurements in the absence of thiamine and are from 6 and 13 independent myocyte preparations for 1 and 2 hour incubations respectively.

A, B, C indicate P<0.025, <0.005, <0.0005 respectively for effects of thiamine (paired tests).

(a)

(b)
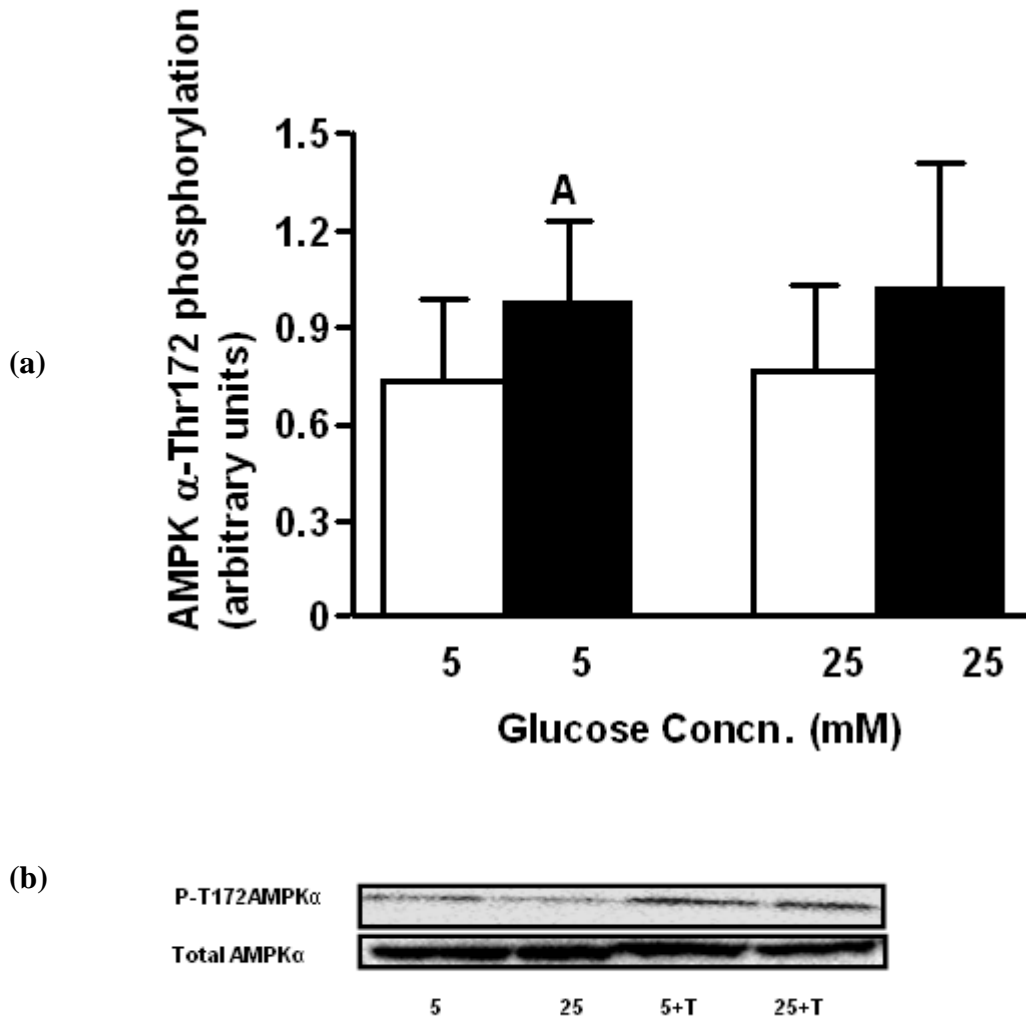
Figure 3.47: the effect of thiamine on the the phosphorylation of Threonine 172 in myocyte AMPK α-subunits. (a) Myocytes were incubated for 2 hours with the indicated concentrations of glucose without (open symbols) or with 1 mM thiamine (filled symbols). Phospho-AMPK measurements were normalized against measurements of total AMPK α-subunit protein. The values are means ± S.E.M. from 4 independent preparations of myocytes. A, indicates a significant (P<0.05) effect of thiamine (paired test). (b) Representative blot.
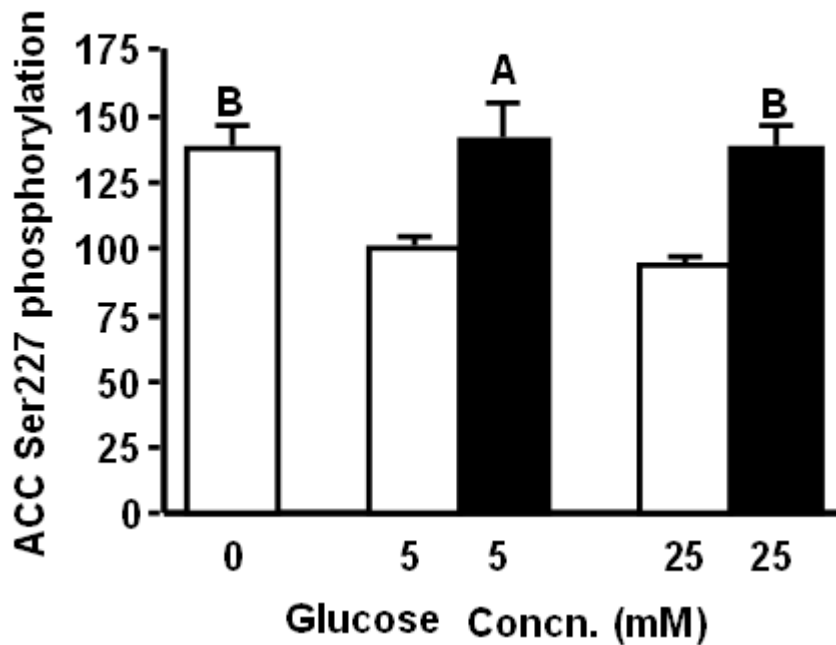
Figure 3.48: the effect of thiamine on the phosphorylation of Serine 227 in myocyte acetyl-CoA carboxylase (ACC). Myocytes were incubated for 2 hours with the indicated concentrations of glucose without (open symbols) or with 1 mM thiamine (filled symbols). Phospho-ACC measurements were normalized against measurements of total ACC protein and then expressed as percentages of the values with of 5 mM glucose in the absence of thiamine. The values are means ± S.E.M. from 5 independent myocyte preparations.

A, B indicate P<0.025, <0.005 respectively versus measurements with 5 mM glucose alone (paired tests).

3.4.8 Conclusion

The above experiments investigated the possibility that glucose or one of its metabolites played a role in driving the AMPK phosphorylation/dephosphorylation system in cardiac myocytes towards inactivation of the kinase. Experiments with 3-O-methylglucose and 2-deoxyglucose suggested that neither glucose itself nor glucose 6-phosphate were likely to be the 'active principle' in this regard. Likewise pyruvate and lactate the terminal products of glycolysis caused no inactivation of AMPK. The lack of any significant effect of azaserine upon AMPK activity also suggested that AMPK activity was unlikely to be affected by an intermediate of the hexosamine pathway. Interestingly AMPK activity was decreased in myocytes incubated with xylitol, a metabolite which in principle can be converted to the PPP intermediate xylulose 5-phosphate an allosteric activator of PP2A. Incubation of cells with ribose which would also be expected to increase the cellular pentose phosphate pool (R5P, Ru5P and Xu5P) failed to decrease AMPK activity. Thiamine and DHA increased AMPK activity whereas PMS had contrary effects. Xylulose 5-phosphate contents increased as both glucose concentrations were increased and in the presence of PMS Wherease DHA decreased xylulose 5-phosphate content.

The curve shown in **Figure 3.43** is only a rough fit to the 6 data points. However it does provide a rough estimate of the xylulose 5-phosphate content which matches half maximal inactivation of AMPK activity. This was approximately 30 nmol per g wet weight of the cells. Interestingly, Mishimura and Uyeda (1995) reported that 10 μM xylulose 5-phosphate gave half maximal activation of a preparation of PP2A from rat liver.

3.9 Assessment of pentose phosphate pathway

Since PMS and DHA were shown respectively to increase and to decrease myocyte Xu5P content the effects of these agents on the oxidation of 5 mM glucose were measured with the aim of determining whether PMS and DHA might have specific effects upon pentose phosphate pathway (PPP) flux quantitated as CO_2 formation by the oxidative arm of the pathway. Effects of thiamine with 5 mM glucose and effects of PMS at the supra-physiological concentration of 20 mM glucose were also investigated.

CO_2 is released from the hexose C-1 position at the 6-phosphogluconate dehydrogenase step in the oxidative arm of the PPP **(Figure 1.6).** If the entire F6P product of the PPP is then swept into glycolytic flux all of the PPP CO_2 is derived from C-1 of the original glucose. By contrast if all of the F6P product of the PPP is recycled to G6P and re-enters the PPP at the glucose 6-phosphate dehydrogenase step the resulting PPP CO_2 is derived from C-1, C-2 and C-3 of the original glucose in equal proportions. No PPP CO_2 is derived from C-4, C-5 and C-6 of glucose. In the glycolysis pathway glucose C-1 and C-6 both become the C-3 carbons of pyruvate and finally give rise to CO_2 in equal proportions in the citric acid cycle. On this basis glycolysis in isolation yields a C-1/C-6 ratio of 1.0 in the metabolites (pyruvate + lactate) and a C-1/C-6 ratio of 1.0 in citric acid cycle CO_2. By contrast when the PPP makes a contribution to glucose metabolic flux the C-1/C-6 ratio found in (pyruvate + lactate) is less than 1.0. Following from this it has been argued that the presence of PPP flux should give rise to a C-1/C-6 ratio of greater than 1.0 in CO_2 and that the size of this ratio provides an index of the relative contributions of the oxidative PPP and glycolysis to glucose catabolism. A related approach has been to take the difference between the yields of $^{14}CO_2$ from [1-^{14}C]glucose and [6-^{14}C]glucose (C-1 minus C-6) as an estimate of metabolic flux in the oxidative arm of the PPP (Hothersall et al, 1979; Muirhead & Hothersall, 1995). This approach was used by Sochor et al. (1984) who observed a positive C-1 minus C-6 value (i.e. a C-1/C-6 ratio of greater than 1.0) in $^{14}CO_2$ when rat heart slices were incubated with 20 mM ^{14}C-labelled glucose.

Burns & Reddy (1977) observed a C-1/C-6 ratio for $^{14}CO_2$ of 0.97 when rat cardiac myocytes were incubated with 5 mM glucose. This ratio was increased to appreciably greater than 1 when competing substrates (pyruvate, lactate, acetate or octanoate) were additionally present. These authors concluded that there can be an appreciable contribution of the oxidative arm of the PPP to glucose catabolism under conditions where mitochondrial oxidation of glucose-derived carbon is suppressed by these other substrates. However two studies with isolated working hearts are at variance with this conclusion. Pfeiffer et al. (1986) using an indirect approach which employed $^{14}CO_2$ washout kinetics could not detect oxidative PPP flux. Goodwin et al. (2001) measured the steady state C-1/C-6 ratios for $^{14}CO_2$ and for ^{14}C-labelled (pyruvate + lactate) and found that both were not significantly different from 1. These authors therefore suggested that normally cardiac oxidative PPP flux, though not necessarily absent, was small compared with overall glucose oxidation.

Table 3.3 shows that incubation of cardiac myocytes with C-1 and C-6 ^{14}C-labelled 5 mM glucose resulted in $^{14}CO_2$ yields per g wet wt. per hour which were broadly similar to those found with heart slices by Sochor et al. (1984). However the C-1/C-6 ratio was not significantly different from 1.0. Interestingly the difference between the yields from C-2 and C-6 labelled glucose was significantly greater than zero. Previously Muirhead & Hothersall (1995) have proposed that a positive value for (C-2 yield minus C-6 yield) is indicative of recycling of fructose 6-phosphate products back into the oxidative arm of the PPP. In an attempt to create conditions where the C-1/C-6 ratio was greater than 1 myocytes were incubated with 5 mM labelled glucose in the presence of physiological concentrations of palmitate or lactate as competing substrates. However, as shown in **Table 3.4** this approach did not lead to a C-1/C-6 ratio of greater than 1. This would appear to contradict the findings of Burns & Reddy (1977). A possible explanation is that the concentrations of competing substrates used in the present experiments were lower than those used by Burns & Reddy (1977). From these experiments I

concluded that a value for the (C-1 yield minus C-6 yield) could not be obtained which gave any sensible index of xidative PPP flux under the chosen experimental conditions. Katz & Wood (1962) have previously discussed the limited quantitative value of the measurement of the C-1/C-6 ratio of $^{14}CO_2$ in the absence of additional measures of glucose utilization.

Following these inconclusive studies I then adopted a different approach to making an estimate of PPP oxidative flux. This approach was based upon the following assumptions. First, in the cardiac myocyte conversion of [U-^{14}C]glucose to $^{14}CO_2$ (the C-U yield) essentially is only due to 6-phosphogluconate dehydrogenase in the oxidative arm of the PPP, pyruvate decarboxylation by pyruvate dehydrogenase (PDH) and the two decarboxylation reactions of the TCA cycle. Second, the yield of $^{14}CO_2$ from [3,4-^{14}C]glucose (C-3/4 yield) provides a measure of the PDH flux. Third, essentially all acetyl-CoA formed by PDH in cardiac myocytes is committed to the TCA cycle. This is a reasonable assumption because fatty acid biosynthesis in these cells is negligible (Awan & Saggerson, 1993). Therefore the value [3 x C-3/4 yield] provides a measure of $^{14}CO_2$ production by PDH and the TCA cycle combined. By difference, $^{14}CO_2$ formation in the PPP then can be calculated as the C-U yield minus the [3 x C-3/4 yield]. This is referred to as the U/3,4-glucose method. It is stressed that this method offers only an approximation to the true PPP oxidative flux because of the following, and probably other, considerations. First, the assumption that the oxidative PPP, PDH and the TCA cycle are the only significant sources of glucose-derived CO_2 may not be totally correct. Second, C-3 from glucose can appear as CO_2 in the oxidative arm of the PPP if fructose 6-phosphate derived from transketolase or transaldolase in the non-oxidative arm of the PPP is recycled back into the oxidative PPP rather than entering glycolysis. Third, transketolase and transaldolase activities put original glucose C-3 into the C-2 position of fructose 6-phosphate. This could decrease the apparent yield of CO_2 at PDH, but at the same time would increase the yield of that carbon as TCA CO_2 (by contrast the original C-4 of glucose remains as the C-4 of fructose 6-phosphate via the PPP or it appears in the C-1 position

of the glyceraldehyde 3-phosphate product of the PPP and the PPP causes no shift in the position of this carbon within pyruvate).

Figures 3.49, 3.51 and 3.53 show that in myocytes incubated with 5 mM glucose alone PPP CO_2 formation was barely detectable by the U/3,4-glucose method. Overall in the series of experiments covered by **Figures 3..49, 3.51 & 3.53** using 21 different myocyte preparations estimates of PPP CO_2 production were 1.5 ± 1.6 µg atoms of carbon per hour per g wet wt. of cells compared with a CO_2 production from [U-^{14}C]glucose of 26.9 ± 3.4 µg atoms of carbon per hour per g wet wt. of cells; i.e. the PPP contributed 5.7% of total glucose oxidation. However the S.E.M. for the PPP CO_2 value was large compared to the mean because 8 out of the 21 calculated values for PPP CO_2 production were negative. The fairest interpretation of these data is that PPP flux from 5 mM glucose cannot be regarded as significantly different from zero. An effect of PMS to increase oxidative PPP flux has previously been shown in mammary gland slices (McLean, 1959) in adipose tissue or in adipocytes (Saggerson & Greenbaum, 1970; Katz & Wals, 1971; Kather et al., 1972), in liver slices (Greenbaum et al., 1971) and in heart slices (Sochor et al., 1984). **Figure 3.49** shows that 30 µM PMS increased total glucose oxidation by 1.9-fold whilst increasing CO_2 formation by PDH plus the TCA cycle by only 1.4-fold. Use of the U/3,4-glucose approximation indicated that 57% of the increase in total glucose oxidation was due to oxidative PPP flux which was increased approximately 19-fold to a value of 14.2 ± 4.1 µg atoms per hour per g wet wt. of cells, a value which is significantly greater than zero (P<0.005, paired test). **Figure 3.50 shows** the effects of 30 µM PMS in myocytes incubated with 20 mM glucose. [14]CO_2 formation from [U-^{14}C]glucose and from [3,4-^{14}C]glucose was increased 2.4-fold and 2.1-fold respectively compared to cells incubated with 5 mM glucose (**Figure3.49**) . **Figure 3.51** shows the effect of 30 µM DHA in myocytes incubated with 5 mM glucose. Total glucose oxidation was significantly decreased by 33% whereas the 9% decrease in [14]CO_2 formation from [3,4-^{14}C]glucose was not statistically significant**.** It should be noted that estimates of PPP CO_2

formation at 5 mM glucose in the absence and presence of DHA differed significantly by 4.8 ± 1.7 µg atoms of carbon per hour per g wet wt. of cells (P<0.005 by paired test) and that this value might be a truer estimate of oxidative PPP flux with 5 mM glucose alone.

Figure 3.52 shows the effects of 30 µM PMS and 30 µM DHA on the yields of $^{14}CO_2$ from C-1 and C-6-labelled 5 mM glucose. Neither agent had any significant effect on the yield of glucose C-6 in CO_2. However PMS significantly increased the C-1 yield 1.8-fold and DHA decreased the same by 38%. Although these data cannot be used quantitatively they qualitatively support the notions that PMS and DHA respectively increase and decrease oxidative PPP flux.

With 5 mM glucose in the presence of 30 µM PMS the C-1 yield was 6.8 µg atoms of carbon per hour per g wet wt. of cells Figure 3.52. This can be compared with an estimated PPP CO_2 production under the same conditions of 14.2 µg atoms of carbon per hour per g wet wt. of cells (Figure 3.49A). Clearly a significant proportion of the PPP CO_2 in the presence of PMS must derive from C-2 and C-3 of glucose after recycling of fructose 6-phosphate units rather than entirely from C-1 of glucose.

Figure 3.53 shows that 1 mM thiamine had no significant effects upon total oxidation of 5 mM glucose or upon $^{14}CO_2$ formation from [3,4-^{14}C]glucose and therefore had no effect upon the estimated low value for PPP CO_2 formation. This was not unexpected as thiamine is a potential precursor for thiamine pyrophosphate (TPP) which is the coenzyme for transketolase, an enzyme of the non-oxidative rather than the oxidative arm of the PPP.

Table 3.3: **The yields of $^{14}CO_2$ by myocytes incubated with C-1, C-2 or C-6 ^{14}C-labelled 5 mM glucose.**

Table 3.3 Myocytes were incubated for 1 hour with C-1, C-2 or C-6 ^{14}C-labelled 5 mM glucose followed by collection and measurement of $^{14}CO_2$. The values are means ± S.E.M. from 12 independent myocyte preparations.

A indicates significantly different from zero (P<0.005 by paired test). N.S. indicates not significantly different from zero (P>0.2).

Glucose label	$^{14}CO_2$ yield (µg atoms per hour per g wet wt. of cells)
C-1	2.98 ± 0.41
C-6	3.10 ± 0.41
C-2	3.61 ± 0.46
C-1 minus C-6	− 0.12 ± 0.17$^{N.S.}$
C-2 minus C-6	+ 0.51 ± 0.13^{A}
C-1/C-6 ratio	1.01 ± 0.06

Table 3.4: The yields of $^{14}CO_2$ by myocytes incubated with C-1 or C-6 ^{14}C-labelled 5 mM glucose in the presence of palmitate or lactate.

Table 3.4 Myocytes were incubated for 1 hour with C-1 or C-6 ^{14}C-labelled 5 mM glucose together with either 0.25 mM sodium palmitate or 1 mM sodium lactate followed by collection and measurement of $^{14}CO_2$. The values are means ± S.E.M. from 4 independent myocyte preparations.

Glucose label	additional substrate	$^{14}CO_2$ yield (µg atoms per hour per g wet wt. of cells)
C-1	Palmitate	3.13 ± 0.61
C-6	Palmitate	4.07 ± 0.93
C-1	Lactate	3.50 ± 0.93
C-6	Lactate	4.23 ± 1.59
C-1/C-6 ratio	Palmitate	0.82 ± 0.10
C-1/C-6 ratio	Lactate	0.96 ± 0.19
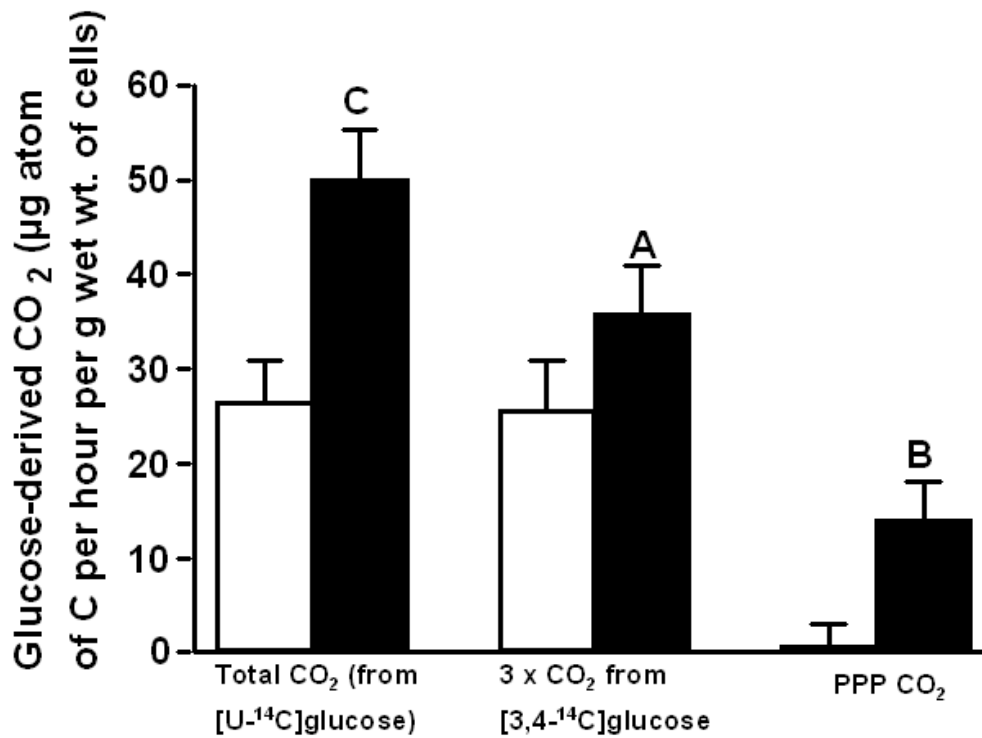
Figure 3.49: the effect of phenazine methosulphate (PMS) on myocyte pentose phosphate pathway (PPP) CO$_2$ formation from 5 mM glucose. Myocytes were incubated for 1 hour with 5 mM glucose labelled with either [U-^{14}C]glucose or [3,4-^{14}C]glucose without (open symbols) or with 30 μM PMS (filled symbols). PPP CO$_2$ formation was estimated by the U/3,4-glucose method. The values are means ± S.E.M. from 12 independent myocyte preparations. A, B, C indicate $P<0.01$, <0.005 <0.0005 respectively for effects of PMS (paired tests).
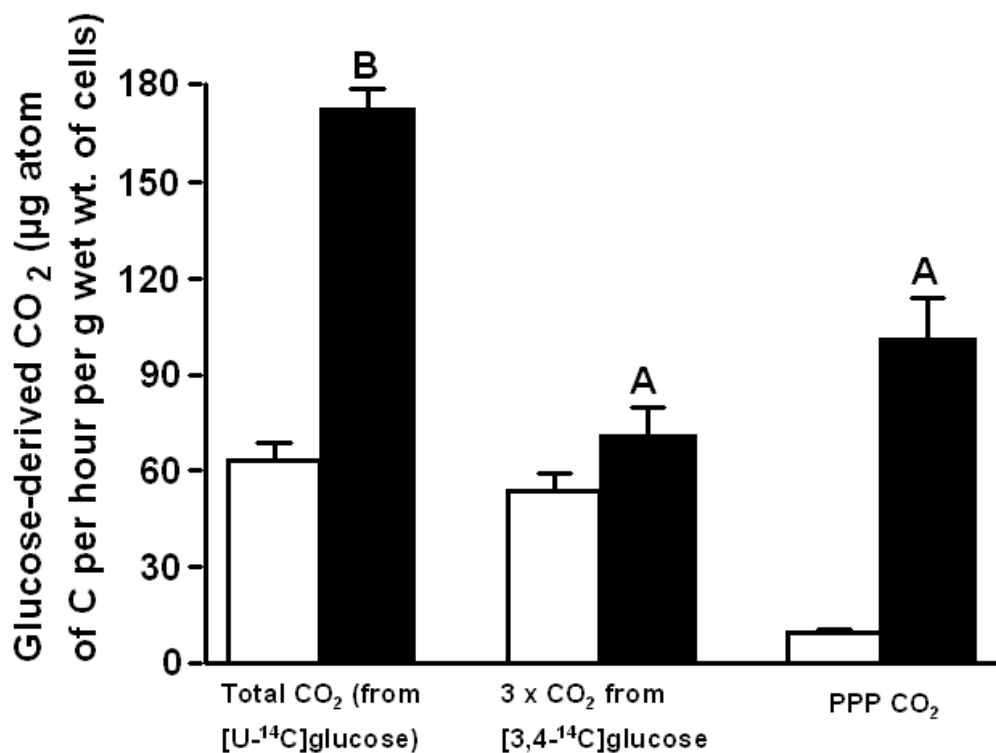
Figure 3.50: the effect of phenazine methosulphate (PMS) on myocyte pentose phosphate pathway (PPP) CO_2 formation from 20 mM glucose. Myocytes were incubated for 1 hour with 20 mM glucose labelled with either [U-^{14}C]glucose or [3,4-^{14}C]glucose without (open symbols) or with 30 µM PMS (filled symbols). PPP CO_2 formation was estimated by the U/3,4-glucose method. The values are means ± S.E.M. from 5 independent myocyte preparations. A, B indicate $P<0.005$, <0.0005 respectively for effects of PMS (paired tests).
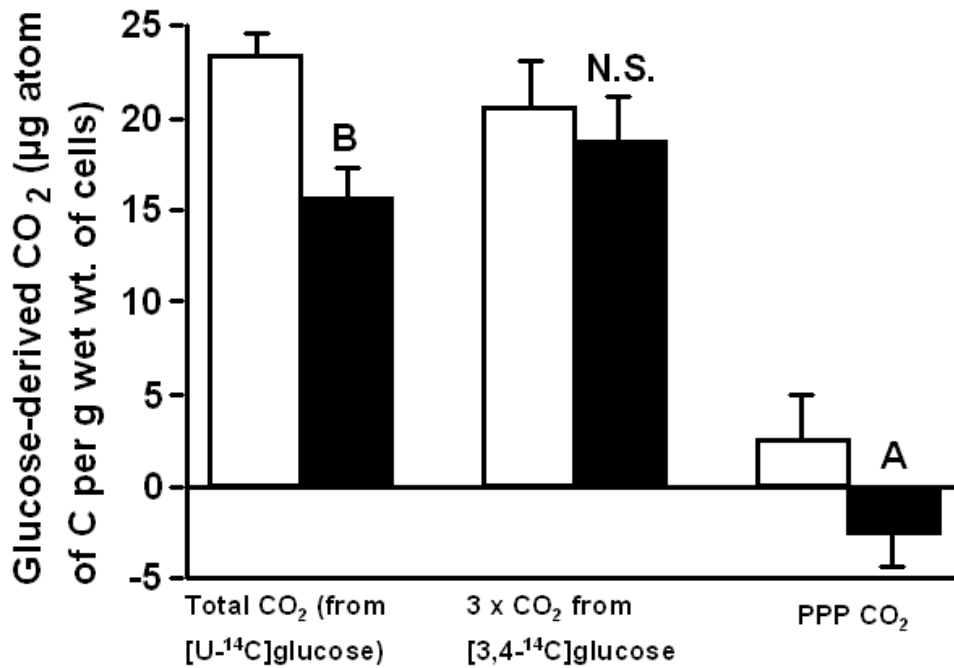
Figure 3.51: the effect of dehydroepi-androsterone DHA on myocyte pentose phosphate pathway (PPP) CO_2 formation from 5 mM glucose. Myocytes were incubated for 1 hour with 5 mM glucose labelled with either [U-^{14}C]glucose or [3,4-^{14}C]glucose without (open symbols) or with 30 µM DHA (filled symbols). PPP CO_2 formation was estimated by the U/3,4-glucose method. The values are means ± S.E.M. from 10 independent myocyte preparations.

N.S., A, B, indicate $P > 0.05$, $P < 0.005$??<0.0005 respectively for effects of DHA (paired tests).
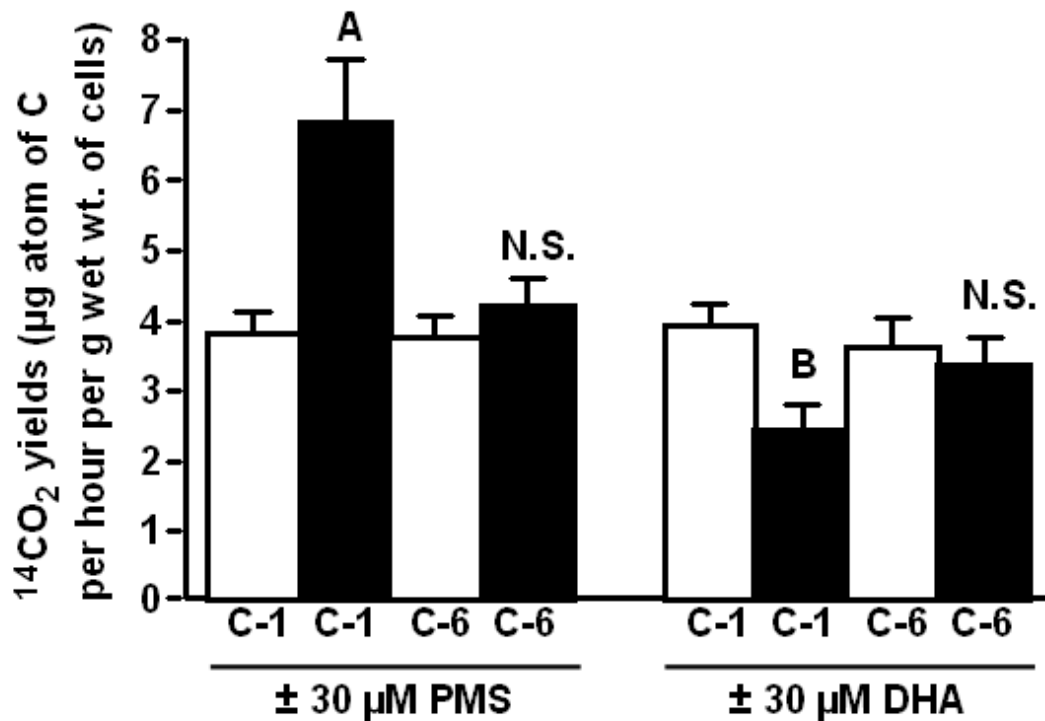
Figure 3.52: **the effects of phenazine methosulphate (PMS) and dehydroepi-androsterone DHA on the yields of CO$_2$ by myocytes incubated with C-1 or C-6 ^{14}C-labelled 5 mM glucose.** Myocytes were incubated for 1 hour with labelled 5 mM glucose without (open symbols) or with PMS or DHA (filled symbols). The values are means $\pm$ S.E.M. from 4 and 7 independent myocyte preparations incubated with PMS and DHA respectively. A, B indicate $P<0.025$, $P<0.005$ respectively for effects of PMS or DHA (paired tests).
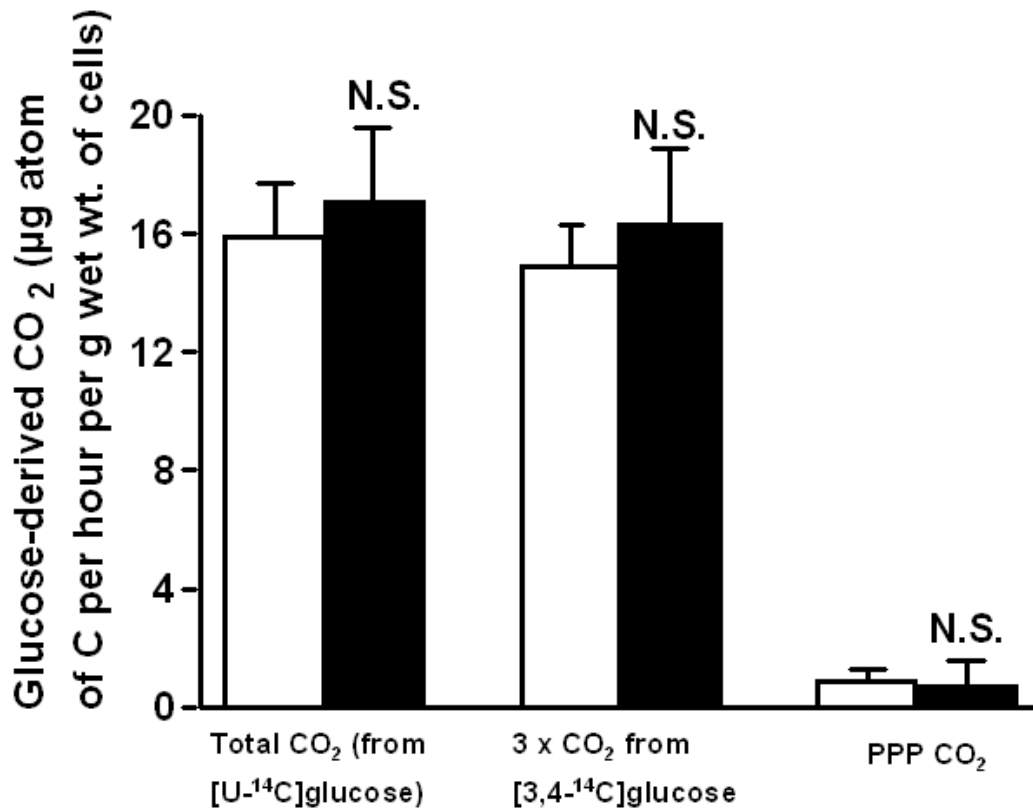
Figure 3.53: **Thiamine had no effect on CO_2 formation from 5 mM glucose by myocytes.** Myocytes were incubated for 1 hour with 5 mM glucose labelled with either [U-^{14}C]glucose or [3,4-^{14}C]glucose without (open symbols) or with 1 mM thiamine (filled symbols). PPP CO_2 formation was estimated by the U/3,4-glucose method. The values are means ± S.E.M. from 5 independent myocyte preparations.

N.S. indicates that thiamine had no statistically significant effects on CO_2 formation (P>0.05 by paired tests).

3.91 Conclusions

I n this study the estimates of the oxidative PPP flux rate gave positive values and the mean rate of formation of CO_2 by the PPP of 9.6 ± 1.3 µg atoms of carbon per hour per g wet wt. of cells was clearly different from zero (P<0.005, paired test). Addition of PMS increased total glucose oxidation and CO_2 formation by PDH plus the TCA cycle by 2.7-fold and 1.3-fold respectively. Additionally PMS had the dramatic effect of increasing oxidative PPP flux by 10.6-fold. 84% of the increase in total glucose oxidation with PMS was attributable to PPP activity. These resuls e strongly suggest that PMS can increase oxidative PPP flux. They also reveal that the oxidative arm of the cardiac myocyte PPP has an enormous reserve capacity.Whereas the mean estimated value for PPP CO_2 production with DHA was negative (-2.57 ± 1.82 µg atoms of carbon per hour per g wet wt. of cells).

This reveals an inaccuracy in the use of the U/3,4-glucose method, but possibly suggests where the real zero value for oxidative PPP flux should be since the myocyte xylulose 5-phosphate content was almost zero in the presence of 30 µM DHA

The observation that thiamine increased AMPK activity (Figure 3.46) suggested the possibility that thiamine would decrease xylulose 5-phosphate content. This could occur provided both (i) a significant proportion of myocyte transketolase normally was in an inactive apo-enzyme form and (ii) transketolase holo-enzyme activity normally was insufficient to equilibrate its reactants thereby rendering the non-oxidative arm of the PPP irreversible. Under those conditions the pentulose phosphate pool (ribulose 5-phosphate and xylulose 5-phosphate) would not be able to be replenished from glycolytic intermediates. The very low content of xylulose 5-phosphate when glucose 6-phosphate dehydrogenase is inhibited by DHA **(Figure 3.43)** would support this notion. Investigation of the effect of thiamine on myocyte xylulose 5-phosphate content should be the subject of further studies.

Chapter-4- General Discussion

4.0 General Discussion

Fatty acids and glucose both play multiple functions in cardiac myocytes extending far beyond their clear use as an energy source. An alternative is to take a less restricted view of metabolism, beyond its stereotypical function as a provider of ATP. Alterations in metabolic flux within cells create essential signals for heart adaptation to each situation. An increasing number of nutrients and hormones are being shown to regulate the AMPK signalling pathway (Xu and Kahn 2006). The work contained in this thesis has allowed a greater understanding of the interaction between glucose, AMPK and the insulin signalling pathway. Once glucose has been transported to the cell and metabolised, it fulfils multiple functions. The original discovery in this study was that, when glucose concentration increases in cardiac myocytes, a significant decrease of α2 AMPK activity. This is associated with a tendency towards a decrease in α-Thr 172 phosphorylation.This work is supported by other work carried out in skeletal muscle which has found a similar outcome(Itani *al et.*; 2003).

Glucose decreased AMPK activity independently of change in the AMP: ATP ratio. No change in total cardiac adenine nucleotide concentration occurred after incubation with 5 or 25 mM glucose in the presence or absence of palmitate. The level of ATP in myocytes obtained in this study 6-8 nmol/mg protein was rather low and the ADP is rather high compared to that found by other groups (see e.g. Ladilov *et al.;* 2003). This might first suggest a technical problem during the analysis of the samples. In order to obtain a clearly visible peak by HPLC, concentrated samples were prepared by extracting the cells with a minimal volume of 0.21 M PCA. It was found difficult to obtain a concentrated neutralised extract in sufficient amount with a higher concentration of PCA because of increased precipitation of $KClO_4$. However, this concentration of PCA is lower than the concentrations commonly used to extract cells, and a previous study measuring the adenine nucleotides using the same method as used in this study reported

incomplete extraction of ATP by 0.21 M PCA (Sellevold *et al.;* 1986). In the unpublished data in our lab adenine nucleotides in the perfused hearts measured in the extract prepared by 0.21M PCA were also approximately half of the values obtained by other groups. Thus the low amount of adenine nucleotides seen in myocytes in this study probably reflects an incomplete extraction of adenine nucleotide.

AMPK activity was increased in the incubations without glucose. Therefore note that this is a totally abnormal state as total adenine nucleotides were decreased suggesting that the cells were so sick that they were catabolised their adenine nucleotides. **Figure 3.10** shows a plot of the effects of glucose at 0, 2, 5, and 25 mM glucose in the presence or absence of palmitate and AICAR, this graph provides evidence that the effect of glucose on AMPK activity could not be adenine nucleotide dependent.

Insulin has previously been reported to inhibit cardiac AMPK via the PI3-PKB pathway (Beauloye *et al*.; 2001). The phosphatidylinositol-3-kinase inhibitor wortmannin is able to overcome the insulin-stimulated decrease in AMPK activity (Beauloye *et al*.; 2001). However, the observation in this study was that PKB was not activated in the absence of insulin and the effect of glucose on AMPK was not mediated by this signalling pathway. The inhibitory effect of insulin is reportedly due to PKB phosphorylation of Ser 485/491 of the α-AMPK α subunit, which prevents the phosphorylation of Thr172 by LKB1 and possibly by other AMPKKs (Horman *et al*.; 2006; Soltys *et al*.; 2006). Recently, this site was also reportedly phosphorylated by PKA *in vitro* (Hurley *et al*.; 2006). However, Ser458/491 phosphorylation was not increased in cardiac myocytes incubated with varying glucose concentrations; this provided evidence that the phosphorylation of AMPK Ser 485/491 is not responsible for the effect of glucose on AMPK activity. Insulin levels decreased overall AMPK activity in hearts perfused with 5 mM glucose (Beauloye *et al*.; 2001) and there appears to be no insulin effect on kinase activity in hearts perfused with 0.4 or 1.2 mM palmitate (Sakamoto *et al*.; 2000). The present study contradicts this

study as it was shown that palmitate had no effect on AMPK inhibition by insulin in cardiac myocytes. AMPK activity was also decreased in newborn rabbit hearts perfused with fatty acids (Makinde *et al*.; 1997) and in adult rat hearts by a ten-fold at higher insulin concentration (Gamble J & Lopaschuk GD, 1997). The ability of palmitate to override insulin dependent increased in malony-CoA and the inhibition of AMPK is also interesting regarding the role of fatty acids in the development of insulin resistance. The elevation of plasma fatty acid concentrations because insulin resistance in human skeletal muscle, an effect associated with decreased IRS-associated PI3 kinase activity (Dresner A *et al*.; 1999).

The role of inositol phosphoglycan molecules as insulin mediators has been postulated mainly on the ability of IPG preparation to mimic the short- and long-term effect of this hormone; which includes modulation of the activity level of many enzymes involved in lipid metabolism, glucose utilisation, phosphorylation and dephosphorylation of cellular proteins, protein synthesis and gene expression and the stimulation of cell growth (Romero & Larner, 1993; Gaulton & Pratt, 1994). Interestingly in cardiac myocytes incubated with 5 or 25 mM glucose in the presence or absence of an IPG preparation AMPK activity was significantly increased at both glucose concentrations by 37% and 82% respectively.

The effect of glucose on AMPK activity was examined in this study to see if it had effects on well-established downstream targets of AMPK such as ACC, eNOS and PFK-2. In this study ACC Ser227 phosphorylation was decreased as glucose concentration increased. In heart AMPK is able to phosphorylate ACCα and ACCβ , resulting in an almost complete loss of ACC activity. ACC in the heart exist in a heterodimeric structure, and some heart ACC associated with the α_2 subunit of AMPK. (Sarah *et al*.; 2001, Lopaschuk *et al.;* 1999). The activity of ACC may 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 increase in malonyl-CoA in response to glucose and glucose plus insulin are associated with an

increase in tissue citrate plus malate (Saha *et al.;* 1997). Purified rat ACCβ is phosphorylated and inactivated in *vitro* by PKA and AMPK (Dyck *et al*.; 1999)

The activation of AMPK in cardiac myocytes increased glucose transport and that this was associated with eNOS phosphorylation at Se1177 (li J *et al.;* 2004). In the present study, phosphorylation eNOS was detected in cardiac myocytes at a very low level even in the presence of AICAR at insulin and all glucose concentrations.

In mice metformin significantly increased the phosphorylation of eNOS Ser1177 phosphorylation over the basal level for a period of 24h (Andrina *et al*.; 2008). Metformin did not alter eNOS Thr495 phosphorylation at any time point investigated. It has been reported that metformin also failed to increase NOS Ser117 phosphorylation when administered to AMPKα2 dn Tg mice (Andrina *et al*.; 2008). Similarly, eNOSThr495 phosphorylation remained unchanged in AMPKα2 dn Tg mice administered metformin.(John *et al*.; 2008). From the above studies eNOS phosphorylation is more detectable after at least two hours, in the present experiments cells were incubated only for 1 hour. This might be the reason that phosphorylation was undetectable even in the presence of AICAR. Another explanation of this discrepancy was that cardiac myocytes are the only cells in the heart that contain GLUT4 (Young *et al*.; 1997), which accounts for much of the glucose transport in the heart tissue. However, in vascular endothelial cells, eNOS is known to be activated by AICAR (Idly *et al.;* 2002); endothelial cells are known predominantly to express the α-1or α-2 isoform of AMPK (Morrow *et al*.; 2003). It has been reported that AICAR activates the α-1 or α-2 isoform and binds to eNOS in the heart muscle, suggesting that AICAR activation of the endothelial cell AMPK and eNOS may play an important role in the stimulation of cardiomyocyte glucose transport through a paracrine mechanism. However, additional immunoprecipitation experiments demonstrated that eNOS was also associated with α2 AMPK, the more predominant isoform in cardiac myocytes, which is virtually absent from endothelial cells (Morrow *et al.;* 2003). Although the cardiac myocyte expression of eNOS is generally low, there is heterogeneity within the heart, with greater eNOS expression in the

specialized endocrinal cells lining of the cardiac chambers (Mungue *et al.;* 2003) and pericardial cardiomyocytes (Brahmajohi & Campell, 1999). Taken together, these findings suggest that both autocrine and paracrine mechanisms may be involved to some extent in the interaction between the AMPK, eNOS and the glucose transport pathways in heart muscle.

 In the heart, AMPK activation leads to the phosphorylation and activation of PFK-2 (Marsin *et al*.; 2000). Elevated glucose levels cause a decrease in AMPK activity, which would subsequently be expected to decrease PFK-2 activity and thereby the cell content of fructose 2, 6-bisphosphate. However, in this study, elevated glucose levels caused an increase in PFK-2 activity. In this circumstance, elevated glucose levels might cause a covalent modification of PFK-2FBPase-2, by a mechanism which overrides any effect resulting from glucose-induced suppression of AMPK, thus leading to the activation of glycolysis. There is substantial evidence to suggest that glucose increases the fructose 2, 6-bisphosphate level. The most convincing lines of evidence come from a demonstration carried out in pancreatic MIN6 cell line. Glucose may have an impact on the fructose 2, 6- bisphosphate content by covalent modification of PFK-2/FBPase or through changes in metabolite concentration that act as activators or inhibitors of either the kinase or bisphosphatase (Catherine *et al*, 2008).

This study provides evidence in support of the hypothesis that glucose metabolites play a role to decrease AMPK activity in cardiac myocytes. Glycolysis metabolites, glycogen and azserine the inhibitor of GFAT had failed to decrease AMPK activity. As a potential mechanism I then focused on the pentose phosphate pathway. Both arms of the pentose phosphate pathway can potentially produce xylulose 5-phosphate which has been shown to induce the expression of several genes in the liver and may modify other signalling pathways, (Doiron B *et al.;* 1996). Cardiac myocytes incubated with xylitol (which is converted to xylulose 5-phosphate) decreased AMPK activity significantly ($p < 0.0005$). In addition the level of xyluose 5-phosphate was

elevated as the glucose concentration was increased. Supporting this finding, xylulose 5-phosphate and AMPK activity in cardiac myocytes show negative correlation.

In this study, AMPK inhibition by glucose was diminished upon G6PD inhibition by the non-competitive inhibitor, dehydroepiandrosterone (DHA). It has been reported that DHA increases Ca^+ levels; however, it appears that changing intercellular Ca^{2+} does not alter AMPK activity in cardiac myocytes (unpublished data in our laborotary). The activation of the pentose phosphate pathway by the artificial electron acceptor PMS resulted in a dramatic decrease in AMPK activity. The pentose phosphate pathway was significantly decreased in the presence of DHA. By contrast, PMS and the increasing glucose concentration significantly increased PPP activity. The accumulating evidence suggests that the pentose phosphate pathway is a crucial regulator of the glucose effect on AMPK activity.

Thiamine reverses the effect of glucose on AMPK activity. This might be through decrease of xylulose 5- phosphate level by accelerating the trasketolase reaction. This may lead to xylulose 5-phospate to be metabolized rapidly and does not accumulate to levels sufficient to activate protein phosphatase 2A.

The mechanism by which Xu 5-P activates protein phosphatase (PP2A) is currently unknown. It has been reported that, in the liver, xylulose-5-phosphate regulates the bifunctional enzyme through PP2A. (Nishimura *et al;* 1995.). This group suggested the possibility is that, since Xu 5-P activates the dephosphorylation of only Fru-6-P, 2-kinase: Fru-2, 6-Pase as a substrate, the pentose-P may bind to the bifunctional enzyme, and the complex could be a better substrate than the free enzyme for the protein phosphatase. Another possibility is that Xu 5-P may bind to the P subunit and dissociate P from the heterotrimer, resulting in activation (Nishimura *et al;* 1995.). Xu 5-P in rat liver represents one of only two examples of metabolite (or small *M*r compound) activation of PP2A. The other is the activation of a heterotrimeric form of PP2A of rat T9 glioma cells by ceramide (Dobrowsky and Hunnun, 1992) (Dobrowsky *et al.;* 1993). However, a more

recent report (Law and Rossle 1995) indicates that the PP2A catalytic subunit also is activated by ceramide. Therefore this ceramide effect is probably unspecific. This is by contrast to the Xu 5-P-activated PP2A, because activation by Xu 5-P shows substrate unspecific, thus reflecting the differences in the activation mechanism. Since PP2A is not only the most abundant form of protein phosphatase, especially in cytoplasm, but also shows broad substrate specificities, it is possible that other PP2As may be regulated by specific metabolites.

4.1 Conclusion

A hypothetical involvement of AMPK in an amplification loop which links cholesterol or fatty acid synthesis with the PPP in a lipogenic tissue such as the liver is shown in (**Figure 4.1**). This amplification loop might be envisaged to function in various situations such as the following.

An increased supply of the substrates for ACC and HMG-CoA reductase (e.g. acetyl-CoA or HMG-CoA) should increase fatty acid/cholesterol synthesis resulting in increased NADPH utilization and hence increased oxidative PPP flux. If this resulted in an increase in the Xu5P content it is envisaged that activation of PP2A would followed by a decrease in AMPK α-Thr172 phosphorylation and activity. In turn this would result in decreased phosphorylation down-regulation of ACC and HMG-CoA reductase leading to further enhancement of these enzymes' activities.

An increase in NEFA supply to the liver, leading to an increase in the cellular content of fatty acyl-CoA, an allosteric inhibitor of ACC, and hence reduce fatty acid synthesis. The resulting decrease in NADPH utilization would lead to a decrease in oxidative PPP flux and possibly a decrease in the content of Xu5P. In this instance operation of the 'Xu5P/PP2A/AMPK axis' described above would lead to activation of AMPK leading to phosphorylation down-regulation of ACC and HMG-CoA reductase activities. Presumably a similar outcome might occur if either fatty acid synthesis or cholesterol synthesis were inhibited pharmacologically. This could possibly provide an explanation for the activation of AMPK by statin drugs (Sun *et al*.; 2006).

A direct activation of AMPK either through an increase in the cellular AMP/ATP or in response to a stimulatory neuroendocrine agent would lead to a decrease in the activities of ACC and HMG-CoA reductase, leading to decreased NADPH utilization, decreased oxidative PPP flux and operation of the 'Xu5P/PP2A/AMPK axis' leading to increased phosphorylation/activation of AMPK and further decreases in the activities of ACC and HMG-CoA reductase. Alternatively, direct inactivation of AMPK either through a decrease in the cellular AMP/ATP or in response to an inhibitory neuroendocrine agent (e.g. insulin) would result in operation of the amplification loop to further decrease the activities of ACC and HMG-CoA reductase.

Presumably these hypothetical amplification effects must reach some upper limit dictated by factors such as the response range of PP2A to Xu5P and the range of phosphorylation/dephosphorylation changes occurring in AMPK, ACC and HMG-CoA reductase. The obvious uncertainties in this hypothesis are (i) the extent to which Xu5P content is altered by oxidative PPP flux and (ii) the important question of whether it is feasible to expect the phosphorylation/dephosphorylation of ACC and HMG-CoA reductase to be linked in this way.
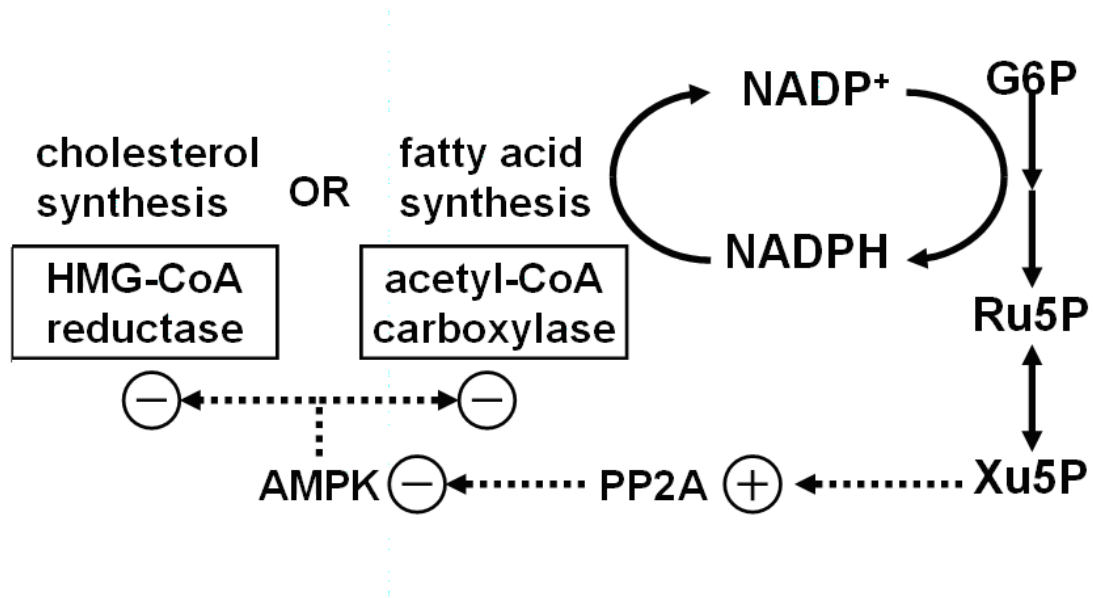
Figure 4.1: The hypothetical involvement of AMPK in an amplification loop linking cholesterol or fatty acid synthesis with the pentose phosphate pathway in a lipogenic tissue such as the liver. See the text for discussion of this hypothesis.

Finally this study has contributed to the ever-increasing knowledge regarding the effect of glucose on AMPK activity. However, in this model, AMPK activation in response to a limited glucose supply presumably has a survival value, because it would both increase ATP generation and decrease the use of ATP for such processes as cholesterol, triglyceride, and possibly protein synthesis as it mention early (Hardie *et al*.; 1997).

Conversely, the decrease in AMPK activity induced by glucose through the pentose phosphate pathway would perhaps direct fatty acids into storage as triglycerides at the expense of oxidation. Hypothetically, it could also allow an up-regulation of other synthetic processes that are inhibited by AMPK.

What is less clear is exactly how this scenario could play out *in vivo,* where blood flow and hormonal (among other) factors might attenuate the effects of hyper- and hypoglycemia on the activity of AMPK. This question could be relevant to such clinical problems as glucose toxicity and the effects of acute hypoglycemia. Finally, AMPK-mediated glucose metabolism could hypothetically act in any cells type.

4.3 Future work

In order to further understand the mechanisms by which AMPK interacts with various nutrients, more studies are needed.

Principally important is the question of how other factors play a role in modulating AMPK activity.

1. Measurements of AMPK activity in heart tissue in response to different substrates after treatment with varying thiamine concentrations.

2. Measurements of xylulose5-phosphate and AMPK activity after perfusion of the hearts with varying glucose concentrations.

3. Measurements of the activity of essential enzymes in the pentose phosphate pathway.

4. Measurements of AMPK activity in the presence of thiamine and hormones.

5. Measurements of pentose phosphate pathway metabolites and flux rates in the presence of AMPK activators.

6. Measurements of AMPK activity in the presence of insulin's second messenger e.g. (IPG) under certain pathological conditions.

Chapter-5-Bibliography

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