Investigations of the Mechanisms Involved in the Regulation of Class II Phosphoinositide 3-kinase.

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by

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Abstract

There is growing evidence that the class-II phosphoinositide (PI) 3-kinases (PI3K) play important roles in regulating cell function with evidence emerging for their role in regulating cellular functions including: clathrin coated vesicle trafficking; nuclear mRNA processing; and the activation of Protein Kinase-B (PKB). The alpha isoform of the class-II PI3K (PI3K-C2α) is activated by a range of growth factors and chemokines, as well as by integrin receptors but the mechanisms responsible for this are poorly understood. As a number of the stimuli known to activate PI3K-C2α are also capable of activating the Protein Kinase C (PKC) and Extracellular Regulated Kinase (ERK) pathways, we investigated whether these could be involved in regulating these aspects of PI3K-C2α biochemistry.

Here we demonstrate that phorbol esters such as Phorbol-12-Myristate-13-Aacetate (PMA) activate PI3K-C2α in different cell types. Using inhibitors of the ERK and PKC cascades we show that phorbol ester-induced activation of PI3K-C2α is ERK-dependent, but PKC-independent; whereas, the insulin-induced activation of PI3K-C2α is independent of ERK but dependent upon other classes of PI3K. Furthermore, we find that PMA and insulin both induce the phosphorylation of PI3K-C2α as shown by bandshifts. The PMA-induced bandshifts were found to be ERK- and PKC-dependent, whereas the insulin-induced bandshift was dependent upon other classes of PI3K. Our studies provide evidence that phorbol esters induce the production of phosphatidylinositol-3-phosphate (PtdIns-3-P), and this is consistent with PtdIns-3-P being produced by PI3K-C2α. We further show that insulin causes PI3K-C2α to associate with two tyrosine phosphorylated proteins one of 160kDa and another of 106kDa, whereas PMA stimulation causes the association of PI3K-C2α with a tyrosine phosphorylated protein of 120kDa. We show that the 120kDa band contains a member of the FAK and p130cas family of focal adhesion protein. We also find that the cytokines leptin and Tumour Necrosis Factor alpha (TNFα) induce an activation of
PI3K-C2α, and this activation is dependent upon ERK, which is similar to that seen with phorbol esters. Our studies further show that adrenaline and insulin are both involved in the regulation of PI3K-C2α in rat soleus muscle and that cAMP analogues activate PI3K-C2α implying that Protein Kinase A (PKA) can also activate PI3K-C2α. In conclusion we propose that there are different pathways involved in the regulation of PI3K-C2α.
Statement

This thesis is an account of research conducted at the Department of Biochemistry and Molecular Biology at University College London, between September 2000 and September 2003. Except where references are given, this thesis contains my own original work, does not exceed the word limit stipulated by the University and is not substantially the same as any I have submitted for any other degree, diploma or examination.

Some of the work presented in this thesis has been published elsewhere:

**Ktori, C., Shepherd P. R., and O’Rourke, L. (2003)** TNFα and Leptin activate the α-isoform of class II phosphoinositide 3-kinase *Biochem. Biophys. Res. Commun.* 306, 139-143


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Abbreviations

Ab  Antibody
BIM-1  Bisindolylmaeimide I
BSA  Bovine Serum Albumin
CHO-IR  Chinese Hamster Ovary cells with Overexpressed Insulin Receptor
DAG  Diacylglycerol
DMEM  Dulbecco Modified Eagle Medium
DMSO  Dimethylsulphoxide
DTT  Dithiothreitol
EDTA  Ethylenediaminetetraacetic acid
EGF  Epidermal Growth Factor
EGFR  Epidermal Growth Factor Receptor
ERK  Extracellular Signal Regulated Kinase
GFP  Green Fluorescent Protein
GST  Glutathione-S-Transferase
IBMX  3-isobutyl-1-methyl-xanthine
IC_{50}  50\% inhibitory concentration
IGF-1  Insulin-like Growth Factor 1
IP_3  Inositol (1,4,5) trisphosphate
IP_4  Inositol (1,3,4,5) tetrakisphosphate
IPTG  Isopropyl-1-thio-β-D-galactopyranoside
IRS-1  Insulin Receptor Substrate-1
JNK  Jun N-terminal kinase
HEK 293  Human Embryonic Kidney cells
LPA  Lysophosphatidic acid
LPL  Lipoprotein lipase
MAPK  Mitogen Activated Protein Kinase
MEK  MAPK Extracellular regulated Kinase
mTOR  Mammalian Target Of Rapamycin
NGF  Nerve Growth Factor
NMR  Nuclear Magnetic Resonance
OD  Optical Density
p70-S6K 70kDa-ribosomal S6 Kinase
p90-RSK 90kDa-ribosomal S6 Kinase
PA Phosphatidic Acid
PAGE Polyacrylamide Gel Electrophoresis
PBS Phosphate Buffered Saline
PDE3b Phosphodiesterase 3b
PDGF Platelet-Derived Growth Factor
PDGFR Platelet-Derived Growth Factor Receptor
PFK-2 Phosphofructokinase-2
PI3K Phosphoinositide 3-kinase
PKA Protein Kinase A
PKB Protein Kinase B
PKC Protein Kinase C
PLC Phospholipase C
PLD Phospholipase D
PMA Phorbol 12-myristate 13-acetate
PH Pleckstrin Homology
PI Phosphoinositide
PTB Phosphotyrosine Binding Domain
PtdCho Phosphatidylcholine
PtdIns Phosphatidylinositol
PtdIns-3-P Phosphatidylinositol-3-phosphate
PX Phox Homology
RTK Receptor Tyrosine Kinase
SCID Severe Combined Immunodeficiency
SDS Sodium Dodecyl Sulphate
SOS Son-of-Severeness
TLC Thin Layer Chromatography
TNFα Tumour Necrosis Factor alpha
Chapter 1  

1. Introduction

1.1 PHOSPHOINOSITIDE LIPIDS AND PI3K

Phosphoinositides (PI) make up a small component of the cell membrane's complement of phospholipids, usually making up less than 10% of the total, which comprises of phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine (Funaki, Katagiri et al., 2000). Phosphatidylinositol (PtdIns) is the building block of PI. The term PI applies to any phosphorylated derivative of PtdIns (Vanhaesebroeck, Leevens et al., 2001; Vanhaesebroeck and Waterfield, 1999c). In cells, all free hydroxyl groups of the inositol ring of PtdIns, apart from those at the 2-OH and 6-OH positions, can be phosphorylated in different combinations (Figure 1.1 Chemical structure of PtdIns).

As many as eight PI species have been identified in eukaryotic cells. Their interconversions are carried out by the action of various kinases and phosphatases (Kanaho and Suzuki 2002) (Figure 1.2 Synthetic pathways for PIs). These include PI3K, PtdIns-4-kinases and PIP kinases (Fruman and Cantley 1998; Stephens, Hughes et al., 1991). PI3Ks phosphorylate inositol lipids at the D3 position of the inositol head group (Fruman and Cantley 1998). Four species of 3-OH phosphorylated PIs have been identified in eukaryotic cells, PtdIns-3-P, PtdIns-3,4-P₂, PtdIns-3,5-P₂ and PtdIns-3,4,5-P₃. PtdIns-3-P, PtdIns-3,4-P₂ and PtdIns-3,4,5-P₃ are generated by the action of PI3Ks (Kanaho and Suzuki 2002) (Figure 1.1 Synthetic pathways for PIs), whereas PtdIns-3,5-P₂ is produced from phosphorylation of PtdIns-3-P by an enzyme known as p235 (PIKfyve), which is the mammalian homologue of the yeast protein Fab1p (McEwen, Dove et al., 1999). 3-
OH phosphorylated PIs other than PtdIns-3-P are barely detectable in resting cells. However, upon cellular stimulation the levels of PtdIns-3,4,5-P_3 and PtdIns-3,4-P_2 rise sharply to a level that is 5-10% that of PtdIns-3-P. PtdIns-3,4,5-P_3 is thought to be generated by direct phosphorylation of PtdIns-4,5-P_2 by class I PI3Ks. A delay in the appearance of PtdIns-3,4-P_2 suggested that this is produced by the action of PI 5-OH-phosphatases on PtdIns-3,4,5-P_3 (Hawkins, Jackson et al., 1992; Stephens, Hughes et al., 1991).

1.1.1 Targets of 3-OH-phosphorylated PIs

Activation of signalling pathways downstream of PI3K is achieved through recruitment of certain proteins containing Pleckstrin Homology (PH), FYVE domains or Phox (PX) Homology domains to the sites where 3-OH phosphorylated PIs are generated. The FYVE domains and PX domains selectively bind PtdIns-3-P, whereas a subgroup of PH domains shows specificity for PtdIns-3,4-P_2 and/or PtdIns-3,4,5-P_3 (Ellson, Andrews et al., 2002). Also, PH domains that bind PtdIns-3-P have been identified recently (Dowler, Currie et al., 2000).

1.1.1.1 The FYVE Domain

The FYVE domain was named after the first four proteins shown to contain it, namely, Fab1p, YOTB, Vac1p, and Early Endosome Antigen 1 (EEA1) (Stenmark, Aasland et al., 1996). The domain consists of 60-80 residues which include eight conserved cysteines that form two separate Zn^{2+} coordination centres. The third cysteine is surrounded by the characteristic basic motif [(R/K)(R/K)HHC] which is involved in
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the binding of the inositol headgroup of PtdIns-3-P (Gaullier, Simonsen et al., 1999; Gaullier, Simonsen et al., 1998). Structural data have shown that only the inositol headgroup of PtdIns-3-P can be accommodated in the FYVE domain (Fruman, Rameh et al., 1999). FYVE domains appear to be less widespread than PH domains and most of them are present in proteins that have been implicated in membrane trafficking.

1.1.1.1 Pleckstrin Homology (PH) Domain

PH domains are globular protein domains of about 100 amino acids and they present low (10-20%) sequence identity to other proteins. PH domains are found in all eukaryotes in more than 100 different proteins. PH domain containing proteins include kinases, phospholipases, adaptor proteins, structural proteins and nucleotide-exchange factors (Lemmon and Ferguson, 2000). Many PH domains have binding capacity for PIs, although the affinity of these interactions varies considerably.

The core PH domain structure consists of two β-sheets, made of four and three β-strands, respectively, which are linked by loop regions which are very variable in sequence and structure (Lemmon and Ferguson, 2000). The β-sheets form a barrel-like structure that is capped on one side by a C-terminal α-helix, which contains a highly conserved tryptophan residue. The inositol head group of the PI ligand is placed between the loops at the end of the barrel. The residues that are essential for binding to PIs reside at the N-terminus in a K-X$_{8-13}$-R/K-X-R-Hyd motif (where X is any amino acid and Hyd is a hydrophobic amino acid). PH domains can be grouped into several classes on the basis of their functional properties (Kavran, Klein et al., 1998). A subset of PH domains bind PtdIns-3,4-P$_2$ and PtdIns-3,4,5-P$_3$ over other PIs (Fruman, Rameh et al., 1999). Most
PH domains that interact with PtdIns-3,4,5-P$_3$ also bind to PtdIns-3,4-P$_2$, although quite often with lower affinity. So far, PH domains that interact only with PtdIns-3,4-P$_2$ have not been identified.

### 1.1.1.2 Phox (PX) Homology Domain

PX domains were firstly identified in two cytosolic components of the NADPH oxidase (Ponting, 1996). PX domains are found in more than 100 known eukaryotic proteins that have distinct functions and include the p40$^{\text{phox}}$ and p47$^{\text{phox}}$ subunits of the NADPH oxidase, class II PI3Ks, cytokine-independent survival kinase (CISK), members of the phospholipase D (PLD) family, sorting nexins (SNX), bud emergence (Bem) proteins and t-SNARE Vam7p. PX domains have been shown to act as specific PI-binding modules (Cheever, Sato et al., 2001; Ellson, Gobert-Gosse et al., 2001) that have lipid binding specificities.

The PX domain is approximately 120 residues long, and sequence comparisons have shown that it contains several well conserved regions, including a number of basic residues and a proline-rich stretch (Ponting, 1996). NMR spectroscopy studies have revealed the overall structure of the PX domain as a three stranded $\beta$-sheet followed by three $\alpha$-helices (Cheever, Sato et al., 2001). The interactions between all PX domains and their bound PIs most likely involve the interaction with 1-phosphate and the inositol ring, as these residues are present in all PIs. The majority of PX domains studied show binding selectivity for PtdIns-3-P, and the importance of this lipid in vesicle trafficking in yeast and mammalian cells has been well described (reviewed Stenmark and Aasland, 1999).
1.1.1.3 C2 domains: Other domains found in signal transduction proteins

Other domains have been identified that confer binding to membrane lipids. One of particular relevance to PI3K signalling is the C2 domain. Most proteins with C2 domains function in signal transduction or membrane trafficking. These proteins include those that are involved in the generation of lipid second messengers, such as phospholipase C (PLCs) and PI3K (PI3K-C2α); in protein phosphorylation, such as PKC; and, in activation of GTPases such as Ras-GAP.

The structure of these C2 domains consists of a compact β-sandwich composed of two four-stranded β-sheets. The three loops at the top of the domain and the four at the bottom connect the eight β-strands. Calcium (Ca²⁺) binding occurs exclusively at the top three loops (Rizo and Sudhof 1998). The Ca²⁺ binding sites are formed by five aspartate side chains, one serine side chain and three carbonyl groups. The Ca²⁺ binding properties of C2 domains confer on them the ability to act as electrostatic switches without requiring conformational changes (Rizo and Sudhof 1998).

1.1.2 Classification of PI3Ks and their mode of action

PI3Ks are a ubiquitously expressed enzyme family capable of phosphorylating the D-3 position of PIs and these enzymes play a crucial role in a wide range of cellular events (see section 1.2) including regulation of metabolism, differentiation, cell growth, apoptosis, vesicle trafficking and cytoskeletal rearrangements (Shepherd, Withers et al., 1998; Toker and Cantley 1997). PI3K was initially purified and cloned as a heterodimeric
complex, consisting of a 110kDa catalytic subunit and an 85kDa-regulatory/adapter subunit. A number of PI3Ks have been identified which all share high homology in the catalytic domain. However, they vary considerably outside this catalytic region and have been sub-classified into class I (which is further sub-classed to Class IA and IB), class II, class III and class IV PI3K (Figure 1.3 Structures of different classes of PI3K) based on shared structural and functional characteristics (Domin, Pages et al., 1997).

1.1.2.1 Class I PI3Ks and their mode of action

Class I PI3Ks are heterodimers made up of a 110kDa catalytic subunit and a regulatory adapter subunit. The class I PI3K enzymes are divided into two subclasses (class IA and class IB PI3K) on the basis of their mechanism of action. The main lipid produced by class I PI3Ks in vivo is PtdIns-3,4,5-P3, however, they are also able to phosphorylate PtdIns, PtdIns-4-P and PtdIns-4,5-P2 in vitro (Vanhaesebroeck, Leevers et al., 1997a).

Class IA PI3Ks are activated by tyrosine-kinase coupled transmembrane receptors (Wymann and Pirola 1998) with different strengths and duration of the signals. For example, some receptors such as the insulin receptor and platelet derived growth factor (PDGF) receptor (PDGFR) activate PI3K strongly, compared to the epidermal growth factor (EGF) receptor (EGFR), which activates PI3K more weakly (Wymann and Pirola 1998). In contrast, p110γ the only known class IB PI3K, and p110β, are activated by G-βγ subunits of heterotrimeric G proteins (Krugmann, Hawkins et al., 1999; Stephens, Eguinoa et al., 1997), which are released in response to activation of seven transmembrane receptors. The p101 adapter subunit in p110γ is essential for the G-βγ
responsiveness of p110γ, but not p110β (Krugmann, Hawkins et al., 1999). Class I PI3Ks (IA and IB) can additionally be activated in vitro and in vivo by GTP-bound (activated) Ras, which interacts directly with a defined domain in the catalytic subunit (Rodriguez-Viciana, Warne et al., 1994).

Class I PI3Ks also have protein kinase activity, p110α can phosphorylate a serine residue (ser 608) in the inter-SH2 domain region of p85α and p85β (Dhand, Hiles et al., 1994); however, recently it has been shown that there are other substrates for p110α (Foukas, Beeton et al., 2004). In comparison p110δ has an autophosphorylation site mapped to the C-terminus, and phosphorylation in vitro of these sites leads to down regulation of the lipid kinase activity of the enzyme (Vanhaesebroeck et al., 1999a; Vanhaesebroeck et al., 1997b).

1.1.2.1.1 Class IA PI3Ks

The class IA PI3K group is a heterodimer consisting of a catalytic subunit (p110) and an adaptor subunit. Mammals have three class IA p110 isoforms that are encoded by three separate genes, these have been termed p110α, β and δ. There are at least seven adaptor proteins generated by expression and alternative splicing of three different genes: p85α, p85β and p55γ (Huang et al., 2001; Hiles, Hu, Mondino et al., 1993; Otsu et al., 1992). p110α and β are widely distributed in mammalian tissue (Hu, Mondino et al., 1993; Hiles, Otsu et al., 1992) however, p110δ is more restricted to leukocytes (Vanhaesebroeck, Welham et al., 1997b).
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1.1.2.1.2 Regulatory/adapter subunit of PI3K

The regulatory adaptor subunit is associated with the catalytic subunit p110. The regulatory adapter subunit (p85) comprises of one SH3 domain, a BH (Breakpoint Cluster Region Homology) domain flanked by two proline-rich regions, one N-terminal SH2 (N-SH2) domain, an inter-SH2 (iSH2) region containing the p110-binding site (Klippel, Escobedo et al., 1993) and one C-terminal SH2 (C-SH2) domain, all of which appear to contribute to the regulation of the enzyme. SH2 domains are commonly found in signal transduction proteins regulating cellular processes (Schlessinger and Lemmon 2003). They are conserved amino acid sequences that have homology to a 100 residue, noncatalytic region of Src tyrosine kinase, and contain binding sites for phosphoryl tyrosine (Bradshaw and Waksman 2002). The SH2 domain allows the p85/p110 heterodimers to bind tyrosine residues that are substrates for receptor-activated tyrosine kinases (RTKs). This interaction is sequence specific, and the SH2 domains of p85 bind to a YXXM motif. This interaction results in a conformational change in the regulatory subunit through to the p110 catalytic subunit, leading to translocation of the cytosolic PI3Ks to the plasma membrane where their lipid substrates and Ras are present, and thus activation of the enzyme (Rodriguez-Viciana, Warne et al., 1994).

1.1.2.1.3 Isoform specific roles of Class I PI3Ks

The presence of multiple isoforms of class IA PI3K suggests specificity in roles. This has been compared in most detail in the case of p110α and p110β, which are expressed in similar sets of tissues. It has been reported that the two widely expressed
class IA PI3K isoforms p110α and p110β differ in their catalytic properties both as lipid and protein kinases (Beeton, Chance et al., 2000).

There is evidence that the functions of p110α and β are different; for example, insulin stimulation causes association of p110β with the GLUT4 glucose transporter compartment (Wang, Bilan et al., 1998), but not association of p110α. Analogous differences occur in signal transduction cascades activated by growth factors such as PDGF and EGFR. For example, p110α is involved in PDGF and EGF-induced mitogenic responses, but not in responses induced by bombesin or lysophosphatidic acid (LPA) (Roche, Koegl et al., 1994). However, p110β in comparison is necessary for insulin and LPA-induced mitogenic responses, but not PDGF-induced responses (Roche, Downward et al., 1998).

Further evidence for the distinct roles of p110α and p110β in platelet-derived growth factor- and insulin-induced actin reorganization in porcine aortic endothelial cells comes from the microinjection of inhibitory antibodies specific for either p110α or p110β (H-Rad, Hajkova et al., 2000). Antibodies against p110α, but not antibodies against p110β, inhibited PDGF-stimulated actin reorganisation, whereas the reverse is true for inhibition of insulin-induced actin reorganisation. Such data indicate that the two PI3K isoforms have distinct roles in signal transduction pathways induced by PDGF and insulin (H-Rad, Hajkova et al., 2000).

1.1.2.1.4 Class IB PI3Ks

To date only one class IB PI3K has been identified, which is the p110γ catalytic subunit associated with a 101kDa regulatory protein (p101); p101 has no sequence
homology to any other known protein (Stephens, Eguinoa et al., 1997). p110γ/p101 heterodimers are activated by G-βγ subunits of heterotrimeric G proteins (Krugmann, Cooper et al., 2002; Stephens, Krugmann, Hawkins et al., 1999; Eguinoa et al., 1997). Data from these studies suggests that p101 and p110γ interact mainly through large areas covering the N and C termini of p101 and the N-terminal half of p110γ. The areas which bind Gβγ giving the major effect on PI3K activity are thought to be located on p101. While modifications of the N terminus of p110γ could modulate its intrinsic catalytic activity, binding to the N-terminal region of p101 was found to be indispensable for activation of heterodimers with Gβγ (Krugmann, Hawkins et al., 1999).

Class IB PI3K appears to be present only in mammals where it shows a restricted tissue distribution, being abundant in blood cells only. This could explain why receptors coupled to G-proteins do not induce PI3K activity in all cell types. Even in leukocytes that have p110γ (Vanhaesebroeck, Welham et al., 1997b), PI3K activity is not always induced upon triggering G-protein-coupled receptors. Therefore generation of PtdIns-3,4-P₂ / PtdIns-3,4,5-P₃ via class IB PI3K is a much less widespread mechanism than the synthesis of these lipids through class IA PI3K.

1.1.2.2 Class II PI3Ks

The class II enzymes have not been widely studied and the available evidence on their functional role in the cell is conflicting. Enzymes of this class are significantly larger than class I or class III PI3Ks, with molecular weights of the order of 200kDa. The class II PI3K differ from class I PI3K structurally, in that they contain a C2 domain at the extreme C-terminus which may act as a phospholipid binding module, enabling calcium
sensitivity (see section 1.1.1.1.3). They also contain a C-terminal Phox (PX) Homology domain (see section 1.1.1.1.2). These domains are involved in regulating class II PI3K activity, and are presumably involved in lipid binding as deletion of the C2 domain increases lipid kinase activity (Arcaro, Volinia et al., 1998). The class II PI3Ks also possess a putative Ras binding domain in the N-terminal region; however at present there is no evidence of class II PI3K binding to Ras. More recently it has been found that PI3K-C2α contains a clathrin-binding domain and that clathrin binds to the N-terminal domain of PI3K-C2α stimulating its catalytic activity towards phosphorylated inositide substrates (Gaidarov, Smith et al., 2001).

Class II PI3Ks were firstly identified in *Drosophila* (Molz, Chen et al., 1996; Macdougall, Domin et al., 1995). *Drosophila melanogaster* and *C. elegans* have a single class II PI3K (Macdougall, Domin et al., 1995) but no class II PI3K has been identified in yeast, *D. discoideum* or plants. To date three mammalian isoforms have been identified: PI3K-C2α, β and γ. PI3K-C2α (Domin, Pages et al., 1997; Molz, Chen et al., 1996; Virbasius, Guilherme et al., 1996) and PI3K-C2β (Arcaro, Volinia et al., 1998; Brown, Ho et al., 1997) are widely expressed, while expression of a third isoform, PI3K-C2γ, is restricted to liver (Misawa, Ohtsubo et al., 1998; Ono, Nakagawa et al., 1998). The *in vitro* substrate specificity of all the class II PI3Ks is similar in that they prefer PtdIns as a substrate but they also phosphorylate PtdIns-4-P under certain conditions (Domin, Pages et al., 1997), however, the *in vivo* product remains unknown.
1.1.2.2.1 Class II PI3Ks and their mode of action in signal transduction

The role of class II PI3Ks in the cell and how they are involved in signal transduction is poorly understood. What is clear though is that a wide range of stimuli can activate the class II enzyme. These include activation by integrins (Zhang, Banfic et al., 1998; Paulhe, Perret et al., 2002), and G-protein coupled receptors such as the chemokine macrophage chemoattractant protein 1 (MCP-1), which induces a transient activation of PI3K-C2α via a Goi-linked mechanism in monocytes (Turner, Domin et al., 1998). Evidence has been presented claiming that the PI3K-C2α is involved in clathrin coated vesicle trafficking (Gaidarov, Smith et al., 2001; Prior and Clague 1999), activation of PKB (Arcaro, Khanzada et al., 2002) and nuclear mRNA processing (Didichenko and Thelen 2001). Expression of PI3K-C2α during Drosophila Melanogaster development (MacDougall, Gagou et al., 2004) has shown that a class II polyproline motif in the catalytic subunit found in Drosophila and C.elegans (Vanhaesebroeck and Waterfield 1999c) (PI3K_68D) can bind the Drk adaptor protein in vitro via the N-terminal SH3 domain of Drk. Drk is the Drosophila homologue of the SH2/S3 domain adaptor protein Grb2 (Olivier, Raabe et al., 1993). This suggests a role for Drk in the localisation of PI3K_68D thus allowing it to modify signalling pathways downstream of cell surface receptors (MacDougall, Gagou et al., 2004).

The mechanism for the regulation of class-II PI3Ks is not clear; indeed mechanisms of activation may differ between that of PI3K-C2α and PI3K-C2β. Additionally both PI3K-C2α and PI3K-C2β have been shown to be downstream signalling targets of activated EGF, PDGF receptors (Arcaro, Zvelebil et al., 2000), and
insulin receptors (Brown and Shepherd 2001; Brown, Domin et al., 1999; Urso, Brown et al., 1999). Surprisingly Insulin-like Growth Factor 1 (IGF-1) does not appear to activate PI3K-C2α despite the fact that insulin and IGF-1 have highly similar signalling pathways (Urso, Brown et al., 1999). Both PI3K-C2α (Didichenko and Thelen 2001; Brown, Domin et al., 1999) and PI3K-C2β (Crljen, Volinia et al., 2002; Brown and Shepherd 2001) undergo a shift in apparent molecular weight that is associated with activation, although it is not yet clear whether these modifications are required for activation. In the case of PI3K-C2α the bandshift appears to be caused by phosphorylation (Didichenko and Thelen 2001; Brown, Domin et al., 1999) while in the case of PI3K-C2β evidence indicates that activation involves a calpain dependent proteolysis step (Crljen, Volinia et al., 2002; Zhang, Banfic et al., 1998). To date there is currently little information on the site(s) or kinase(s) involved in this phosphorylation. Recent evidence indicates that phosphorylation of PI3K-C2α is involved in regulating proteolytic degradation of the enzyme rather than an activation of the enzyme (Didichenko, Fragoso et al., 2003). These reports showed that PI3K-C2α is phosphorylated on Ser259 after UV irradiation, and that this phosphorylation was dependent on the activation of c-jun N-terminal Kinase (JNK) signalling pathways, but independent of ERK or the p38 MAPK family of enzymes. To date there are no reports of targetted deletion or RNAi approaches to reduce expression of PI3K-C2α. If such a system was available it would help in the elucidation of the signalling mechanisms involved in the regulation of PI3K-C2α and thus the function of PI3K-C2α.
1.1.2.3 Class III PI3Ks

Class III PI3Ks are the homologues of the yeast vacuole protein-sorting mutant *VPS34* (Stack, Horazdovsky et al., 1995). These PI3Ks can use PtdIns as a substrate *in vitro* and *in vivo*, and are responsible for producing PtdIns-3-P in cells. Vps34p associates with Vps15p (a serine/threonine kinase required for activity of Vps34p) in yeast and p150 in mammals, a Ser/Thr protein kinase that is myristoylated at the N-terminus and has a role in protein trafficking to the vacuole (Stack, Horazdovsky et al., 1995).

Class III PI3K has been shown to be involved in a variety of vesicular trafficking events. For example, Vps34p, the yeast class III PI3K is essential for vesicular trafficking from the golgi to the vacuole (Corvera and Czech 1998). In yeast, Vps34p forms a heterodimer with the serine/threonine kinase Vps15p and regulates vesicle trafficking through proteins containing FYVE finger domains that can bind PtdIns-3-P.

PI3K is a known regulator of phagocytosis; for example, recent reports demonstrate that class I and class III PI3Ks act consecutively in phagosome formation and maturation, and that their respective products, PtdIns-3,4,5-P$_3$ and PtdIns-3-P, accumulate transiently at different stages (Gillooly, Simonsen et al., 2001; Vieira, Botelho et al., 2001).

1.1.2.4 Class IV related PI3Ks

The class IV PI3Ks comprise of a number of proteins which share the conserved catalytic domain of the PtdIns-3- and PtdIns-4-kinases. They include a group of gene products that control cell cycle progression in response to DNA damage which consists of RAD3, MEC1, TEL1, ATM (ataxia telangiectasia mutated) and ATR (ataxia
telangiectasia and Rad3-related), the DNA-activated protein kinase (DNA-PK) and the TOR/FRAP/RAFT1 (target of rapamycin) (Hoekstra 1997). Most of them possess Ser/Thr kinase activity (ATM, ATR, DNA-PK, TOR), but no lipid substrates have been identified for this class of enzymes to date (Jung, Kondratyev et al., 1997; Hartley, Gell et al., 1995).

1.2 BIOLOGICAL ROLES OF PI3K SIGNALLING

Extensive studies have been carried out on the functional characterisation of PI3Ks after growth factor and hormone stimulation. The physiological consequences of enzyme activation include cell growth, survival, differentiation, proliferation, vesicular trafficking, cytoskeletal rearrangements, cellular mobility and metabolism.

1.2.1 PI3Ks and cell growth

A number of reports indicate that PI3K plays a critical role in growth factor signalling to cell growth and proliferation in Drosophila. Overexpression of the Drosophila class IA ortholog, Dp110, in wing or eye imaginal discs resulted in flies with enlarged wings or eyes. Also overexpression of Dp110 containing a mutation that has been predicted to lead to loss of catalytic activity resulted in smaller wings and eyes. These changes are due to changes in both cell size and cell number (Leevers, Weinkove et al., 1996).

Further to these studies, the role of Dp110 and its adaptor, p60, in the control of imaginal disc cell size, cell number and organ size have been investigated. Null mutations in Dp110 and p60 were generated and used to demonstrate that they are essential genes
required for imaginal disc cells to achieve their normal adult size. Modulating Dp110 activity resulted in either the increase or decrease cell size in the developing imaginal disc (Weinkove, Neufeld et al., 1999).

There is also evidence for a similar role of PI3K in mammals. It has been demonstrated that expression of a constitutively active p110α in cardiac myocytes increases the size of the organ (Shioi, Kang et al., 2000). Conversely, expression of a dominant negative version reduces the size. Again, these effects on heart size were accompanied by parallel effects on myocytes size. However, it should be noted that normal heart grows through increase in the size of cardiomyocytes without division, whereas in other tissues of mammals growth is achieved by an increase in cell number (Conlon and Raff 1999).

1.2.2 PI3Ks and DNA synthesis/Cell division/Cell cycle control

Control of DNA synthesis is another process where PI3K has been implicated. Mutation of class I PI3K binding sites on the PDGF receptor resulted in inability of PDGF to induce DNA synthesis (Valius and Kazlauskas 1993). Consistently, inhibition of PI3K by wortmannin and LY294002 (see section 1.6), or by microinjection of neutralizing antibodies had the same effects (Vanhaesebroeck, Jones et al., 1999b; Roche, Downward et al., 1998).

More recently reports demonstrate a specific role of PI3K enzymes in human colon cancer cell growth (Benistant, Chapuis et al., 2000). Reports show that PI3Kα and PI3Kβ play important roles in human colon cancer cell growth with a specific function.
for PI3KB in de novo DNA synthesis and an involvement of PI3Kα in cell survival (Benistant, Chapuis et al., 2000). The finding that activated PI3K can promote entry into the cell cycle suggests that constitutive activation of PI3K could lead to uncontrolled cell growth such as that observed in cancer.

1.2.3 PI3Ks and apoptosis

It is generally thought that PI3K exerts an antiapoptotic role; this view has been formed as a consequence of experimental data showing that inhibition of PI3K induces apoptosis. Conversely, activation of PI3K has been shown to delay apoptotic cell death (Khwaja, Rodriguez-Viciana et al., 1997; Kulik, Klippel et al., 1997). However, in p110α knockout mice a proliferative defect, but not an increase in apoptosis is evident (Bi, Okabe et al., 1999). Furthermore, reports show that Drosophila larvae that are deficient of class I PI3K can live normally for up to 20-days (Weinkove, Neufeld et al., 1999). Conversely, however it has been demonstrated that over activation of PI3K can promote apoptosis. Expression of an inducible p110α in fibroblasts resulted in abnormal entry into the S phase, cell cycle arrest and in the absence of serum in apoptosis (Klippel, Escobedo et al., 1998).

With respect to the molecular mediators of these effects, it seems that PKB is a key molecule. PKB delays cell death in a variety of cell types upon different apoptotic stimuli (Downward 1998; Franke, Kaplan et al., 1997). Several mechanisms have been proposed to explain PKB actions in this pathway. It has been shown that PKB directly phosphorylates BAD (Bcl-2/Bcl-X\textsubscript{L}-Antagonist, causing cell Death), a protein that binds the anti-apoptotic proteins Bcl-2 or Bcl-X\textsubscript{L}, thus preventing their anti-apoptotic function.
Phosphorylation of BAD results in its binding to 14-3-3 proteins, thus releasing Bcl-2/Bcl-X\textsubscript{L} and allowing them to exert their antiapoptotic effects (Downward 1999). Regulation of Caspase-9 activity has also been proposed as an effector of PKB action. Caspase-9 is a protease that plays a key role in the initiation of the apoptotic processes. Direct phosphorylation and inactivation of human caspase-9 by PKB has been reported (Cardone, Roy et al., 1998). However, the significance of this mode of regulation is unclear at the moment.

Another potential mode of anti-apoptotic function of PKB is via phosphorylation of transcription factors such as the members of the forkhead (FH) family and the NF-\text{kB}. Phosphorylation of the FH transcription factors by PKB renders them inactive by preventing their entry into the nucleus. FH transcription factors have been implicated in the transcription of the Fas ligand (Brunet, Bonni et al., 1999). Fas ligand is a well-known inducer of apoptosis that is produced by autocrine and paracrine mechanisms and then binds and activates death receptors. Thus, inhibition of the FH activity after phosphorylation by PKB would decrease production of the Fas ligand and consequently initiation of apoptotic signalling. Similarly, PKB has been implicated in the regulation of the transcription factor NF-\text{kB} activity. In this case, the mechanisms of activation are controversial at the moment (Delhase, Li et al., 2000). However, it seems possible that induction of anti-apoptotic genes such as inhibitor-of-apoptosis proteins (IAPs) might confer a mode of regulation (Wang, Pentyala et al., 1999; Wang, Bilan et al., 1998).
1.2.4 PI3Ks and the actin cytoskeleton

There is evidence that PI3K is involved in the acute regulation of the cytoskeleton by growth factors. For example, reports show that microinjection of recombinant p85α (regulatory subunit of PI3K) into MTLn3 cells blocks both EGF-stimulated mitogenic signalling and lamellipod extension (Hill, Huang et al., 2001). In contrast, a truncated p85, which lacks the SH2 and iSH2 domains and does not bind p110, had no effect on EGF-stimulated mitogenesis but still blocked EGF-stimulated lamellipod extension. Deletional analysis showed that the SH3 domain was not required for inhibition of lamellipod extension, as a construct containing only the proline-rich and breakpoint cluster region (BCR) homology domains was sufficient for inhibition. Overall, this report suggests that proline-rich and BCR homology domains of p85 are involved in the coupling of p85/p110 PI3Ks to the regulation of the actin cytoskeleton (Hill, Huang et al., 2001).

Reports show that PDGF beta-receptor activation of membrane ruffling is inhibited by three independent manipulations of PI3K activity: firstly, by the deletion of tyrosine residues in the PDGF beta-receptor to which PI3K binds, secondly, by the overexpression of a mutant 85kDa PI3K regulatory subunit to which the catalytic kinase subunit cannot bind; and thirdly, by the addition of wortmannin, a potent inhibitor of the catalytic activity of PI3K. This report suggests that PtdIns-3,4,5-P₃ synthesis is required for growth-factor-stimulated membrane ruffling in porcine aortic endothelial cells, and that this might be a part of a signalling pathway leading to direct or indirect activation of the small GTP-binding protein Rac (Wennstrom, Hawkins et al., 1994).
Cell migration represents an important cellular response that uses cytoskeletal reorganisation as its driving force. Reports describe a new signalling cascade, linking PDGF receptor stimulation to actin rearrangements and cell migration. They demonstrate that PDGF activates Cdc42 and its downstream effector N-WASP to mediate filopodia formation, actin stress fibre disassembly, and a reduction in focal adhesion complexes, that is independent of PI3K enzymatic activity, but dependent on p85α, the regulatory subunit of PI3K. Such observations are important for p85α in controlling PDGF receptor-induced cytoskeletal changes and cell migration. (Jimenez, Portela et al., 2000).

1.2.5 PI3Ks and vesicular trafficking

PI3Ks have been linked to membrane trafficking processes including exocytosis, endocytosis and vesicle trafficking (Backer 2000; Wymann and Pirola 1998; Shepherd, Reaves et al., 1996). The first evidence implicating 3-OH-phosphorylated PIs with endocytic machinery came from experiments where mutations impairing PI3K association with PDGF receptor interfered with the trafficking of the receptor to the lysosome (Joly, Kazlauskas et al., 1994). Although the precise mechanisms through which PI3Ks mediate their effects on endocytosis have not been identified, these functions have been attributed predominantly to class III PI3Ks. The archetypal class III PI3K, the yeast PI3K Vps34p, was identified as a mutant with defective protein sorting to the vacuole (Backer, 2000). The function of Vps34p (and of its mammalian ortholog hVps34) and its product PtdIns-3-P became clear after the identification of the FYVE domain-containing proteins as effectors of PtdIns-3-P. A key finding was the identification of the endosomal protein EEA1 (Early Endosomal Antigen 1) as a critical
regulator of endosomal fusion in mammalian cells. EEA1 contains a Rab5-binding domain and a FYVE domain that binds to PtdIns-3-P produced by Rab5-associated hVps34 (Murray, Panaretou et al., 2002). Once recruited to the early endosome via the Rab5/Vps34, EEA1 is thought to mediate both tethering of adjacent endosomal vesicles, and recruitment of components of the vesicular fusion machinery.

Links between PI3K and vesicle trafficking have been best characterised in the case of class III PI3K. A range of vesicle trafficking pathways in the cell rely on formation of clathrin coated pits and there is clear evidence that PI3K is required for this process. However, the full details of the mechanisms involved have not been elucidated. Recent evidence points to a role for PI3K-C2α in this process (Gaidarov, Smith et al., 2001).

One of the most intensely studied vesicle trafficking events is the insulin-induced translocation of GLUT4 transporters. Translocation of the GLUT4 glucose transporters to the plasma membrane was completely abolished by nanomolar concentrations of wortmannin (Kanai, Ito et al., 1993) thus providing the first evidence for the involvement of PI3K in this process. Low nanomolar concentrations of both wortmannin and LY294002 have been shown to block the insulin-stimulated increase in the rate of GLUT4 exocytosis, with little effect on the rate of endocytosis (Clarke, Young et al., 1994). However, more recent evidence has been presented that there are two separate wortmannin-sensitive sites in GLUT4 translocation: one which is blocked by low nanomolar concentrations of wortmannin, and another requiring higher concentrations of wortmannin, suggesting roles for different PI3K isoforms at different steps (Maffucci, Brancaccio et al., 2003).
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A role for PI3K has also been demonstrated in phagocytosis (Araki, Johnson et al., 1996). Interestingly, it has been shown that both class I and class III PI3K participate in this process. Class III PI3K is essential for both formation and maturation of the phagosome, whereas class I PI3K is required for optimal phagocytosis but not maturation (Vieira, Botelho et al., 2001). Further, PI3Ks have also been implicated in the trafficking of the late endosomes. Once again, several proteins containing FYVE domains have been identified as taking part to this process and they are likely to be effectors of Vps34p.

1.2.6 PI3Ks and metabolism

PI3K has been implicated in the regulation of several metabolic processes (Shepherd, Withers et al., 1998), one of them being glycogen synthesis which in a way complements PI3Ks role in glucose transport. Reports show that overexpression of constitutively active PKB results in increased glycogen synthase (GS) activity in L6 myotubes (Ueki, Yamamoto-Honda et al., 1998). However contradictory results have been obtained and it seems more likely that alternative pathways lead to glycogen synthase activation by insulin. There is evidence that JNK regulates glycogen synthesis via Glycogen Synthase Kinase-3 (GSK3) (Moxham, Tabrizchi et al., 1996). Also, rapamycin has been show to attenuate GS and glycogen synthesis in muscle and 3T3-L1 adipocytes without inhibiting insulin-stimulated glucose transport (Cross, Watt et al., 1997; Shepherd, Nave et al., 1995)

The role of PI3K on glucose transport has been studied extensively. It has been shown that insulin is capable of controlling metabolic functions such as the stimulation of
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glucose uptake, and glucose transporter (GLUT4) translocation in muscle and adipose cells. For example, reports show that in L6 skeletal muscle cells wortmannin significantly inhibits the basal PI3K activity, decreases the levels of PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 and abolishes the activation of PI3K by insulin (Tsakiridis, McDowell et al., 1995). Wortmannin inhibited the basal rate of transport of glucose and of amino acids into the cell and abolished their stimulation by insulin. In unstimulated cells, wortmannin decreased GLUT4 glucose transporter content at the plasma membrane, and prevented insulin recruiting transporters to this membrane (Tsakiridis, McDowell et al., 1995). The intracellular pools of the GLUT3 and GLUT4 glucose transporters were reduced in the presence of wortmannin (Tsakiridis, McDowell et al., 1995).

PI3K activation has also been shown to play a role in the regulation of many enzymes involved in glucose metabolism for example, phosphofructokinase-2 (PFK-2). Reports show that in cardiac muscle, insulin can stimulate glycolysis by phosphorylating and activating PFK-2. This activation was found to be inhibited by the classic PI3K inhibitor wortmannin, indicating that PI3K is required in this process (Lefebvre, Mechin et al., 1996). Furthermore to support the finding that PI3K is involved, PKB was found to phosphorylate and activate PFK-2 in vitro (Deprez, Vertommen et al., 1997).

In addition to glucose metabolism, PI3K has been implicated in signal transduction pathways by which insulin regulates both the synthesis and degradation of triacylglycerols. Insulin exerts an antilipolytic effect by inactivating hormone-sensitive lipase (HSL). Reports demonstrate that PI3K inhibitors prevent inactivation of HSL, thus blocking the antilipolytic effect of insulin (Okada, Kawano et al., 1994; Rahn, Ridderstrale et al., 1994). HSL is activated following phosphorylation by PKA (Stralfors
Regulation of HSL activity by insulin is dependent on phosphorylation and activation of phosphodiesterase 3b (PDE3b), which leads to a decrease in levels of cAMP and PKA-induced phosphorylation, and then, in turn, activation of HSL. Reports demonstrate that PI3K inhibitors block the insulin-stimulated phosphorylation of PDE3b (Rahn, Ridderstrale et al., 1994), suggesting that PI3K be involved in this process. Finally, PI3K has also been implicated in the production and secretion of very-low-density lipoprotein by the liver. PI3K inhibitors have been found to prevent insulin from inhibiting apolipoprotein B incorporation into very-low-density lipoprotein (Phung, Roncone et al., 1997).

1.2.7 PI3Ks and regulation of protein synthesis

Various growth factors regulate protein synthesis in a manner that involves co-ordinated regulation of amino acid uptake, gene transcription and mRNA translation and PI3K has been shown to mediate many of these processes. PI3K inhibitors have been shown to block insulin-stimulated uptake of certain amino acids (Tsakiridis, McDowell et al., 1995).

However, the acute effects of growth factors on protein synthesis are mainly mediated by increases in the rate of translation of existing mRNAs (Proud and Denton 1997). The rate of translation is regulated by the functional state of the initiation and elongation complexes. PI3K has been shown to influence this process in several ways. The eukaryotic initiation factor 2b (eIF2b) becomes dephosphorylated and activated in response to insulin as a consequence of activation of the PI3K-PKB-GSK3 axis (Welsh, Stokes et al., 1997). Also, insulin induces dephosphorylation and activation of the...
eukaryotic elongation factor-2 (eEF2), and this leads to an increase in translation elongation. This process has been shown to be both wortmannin and rapamycin-sensitive, since these inhibitors block insulin’s inhibitory effect on a kinase upstream of eEF2 (Redpath, Foulstone et al., 1996). Furthermore, insulin stimulates the phosphorylation of eukaryotic initiator factor 4E (eIF4E) binding protein-1 (4EBP1 or PHAS-I). eLF4E is a constituent of a complex mediating the initiation of translation of an mRNA subset termed 5’-cap-mRNAs. In resting cells, 4EBP1 binds eIF4E preventing it from taking part in the formation of the translation initiation complex. Upon agonist stimulation, 4EBP1 becomes phosphorylated and this causes dissociation of the eIF4E/4EBP1 complex thus relieving the inhibition of translation of the 5’-CAP mRNAs (Diggle, Moule et al., 1996; Manteuffel, Gingras et al., 1996). Multiple phosphorylation sites exist on 4EBP1 and they have been shown to be both wortmannin-and rapamycin-sensitive, thus involving PI3K and mTOR in the regulation of these phosphorylations.

1.3 INSULIN SIGNAL TRANSDUCTION

Insulin is a hormone produced by the β-pancreatic islet cells in response to elevated levels of nutrients, such as glucose, in the blood supply. Insulin binds to its receptor in insulin-responsive tissue for example skeletal muscle, adipose tissue and liver. This then leads to both, the activation of signalling pathways, such as transport of nutrients for example glucose, amino acids and fatty acids; and, the conversion of these nutrients into storage macromolecules such as glycogen, proteins and lipids. The inability to carry out these two processes after feeding leads to Type 2 diabetes. Type 2 diabetes is late onset and usually occurs in adults. The target tissue becomes resistant to the effects
of insulin. Drug intervention, and nutritional control treat this type of diabetes; although the spectrum of drugs available are of poor efficiency and patients usually suffer long-term complications such as kidney, heart disease and loss of sight.

1.3.1 Insulin Signalling at the plasma membrane

Insulin binding to its receptor results in receptor autophosphorylation on tyrosine residues, and the tyrosine phosphorylation of the insulin receptor substrates (IRS-1, IRS-2 and IRS-3) by the insulin receptor tyrosine kinase. This then allows the association of IRSs with the regulatory subunit of PI3K via SRC homology 2 (SH2) domains in the p85 regulatory subunit of class IA PI3K. This results in the recruitment of PI3K to the plasma membrane bringing it in close proximity of its physiological substrate PtdIns-4,5-P$_2$, which it phosphorylates on the D-3 position of the inositol ring to produce PtdIns-3,4,5-P$_3$ (Figure 1.4 Insulin stimulation of PI3K). PI3K activates PtdIns-3,4-P$_2$/PtdIns-3,4,5-P$_3$-dependent kinase 1 (PDK1), which like PKB has a PH domain capable of binding PtdIns-3,4,5-P$_3$. PKB, a serine kinase has a PH domain located at its amino terminus, which binds to PtdIns-3,4,5-P$_3$ and PtdIns-3,4-P$_2$. PDK1 phosphorylates PKB and this interaction at the plasma membrane brings PKB in close contact with other protein kinases that phosphorylate PKB. PKB once activated dissociates from the plasma membrane and phosphorylates substrates in the cytoplasm and nucleus. One of these is another protein kinase GSK-3. A key GSK-3 substrate is GS, which catalyses the final step in glycogen synthesis, the conversion of UDP-glucose into glycogen. Phosphorylation of GS by GSK3 inhibits GS. PKB phosphorylates GSK-3 resulting in the inactivation of the enzyme. Overall inactivation of GSK-3 by PKB results in
déphosphorylation of GS through the action of protein phosphatases and hence the activation of glycogen synthesis.

### 1.3.2 Insulin signalling and glucose uptake

A key action of insulin is to stimulate glucose uptake into the cells by inducing the translocation of the glucose transporter, GLUT4, from the intracellular storage to the plasma membrane sites (Saltiel and Kahn 2001). Activation of PKB leads to translocation of GLUT4 vesicles from their intracellular pool to the plasma membrane, where they allow uptake of glucose into the cell.

Overexpression of constitutively activated mutants of PI3K and PKB stimulate the recruitment of GLUT4 to the cell surface in the absence of insulin. More recently Saltiel and colleagues have reported that another PI3K independent pathway may provide a second signal which allows insulin to stimulate the recruitment of GLUT4 to the plasma membrane. In this pathway activated insulin receptor directly phosphorylates the proto-oncogene Cbl, which activates the TC10 family of Rho GTP-binding proteins. It is thought that these proteins interact with unknown effector proteins to allow insulin-stimulated GLUT4 translocation (Saltiel and Kahn 2001).

### 1.3.3 Insulin stimulation of other signalling pathways

Other signal transduction proteins interact with IRS molecules, including Grb2 and SHP2. SHP2, is a protein-tyrosine phosphatase (PTP) containing SH2 domains. Grb2, is a adaptor protein which contains SH3 domains and allows constitutive association with the guanine nucleotide exchange factor mSOS, and is part of the cascade
including RAS, RAF and MEK that leads to activation of mitogen activated protein kinase (MAPK), and mitogenic responses such as gene transcription stimulated by FOS and ELK1.

Insulin stimulation of MAPK/ERK requires the tyrosine phosphorylation of IRS proteins, and Shc, another substrate of the insulin receptor that contains a SH2 and phosphotyrosine binding (PTB) domain. Tyrosine phosphorylation of IRS or Shc leads to interaction with the adaptor protein Grb2, recruiting the Son-of-Sevenless (SOS) exchange protein to the plasma membrane for activation of Ras. Activation of Ras also requires stimulation of the tyrosine phosphatase SHP2, through its interaction with receptor substrates such as Gab-1 or IRS1/2. Once activated, Ras acts as a molecular switch, stimulating a serine kinase cascade through activation of Raf, MEK and ERK. Activated ERK can translocate into the nucleus initiating transcription that leads to differentiation and cellular proliferation.

Signal transduction by the insulin receptor is not limited to its activation at the cell surface. The activated ligand receptor complex, at the cell surface, is internalised into endosomes. This process is dependent on tyrosine autophosphorylation. Endocytosis of activated receptors has the dual effect of concentrating receptors within endosomes and allowing the insulin receptor tyrosine kinase access to substrates.
1.4 PKC AND PHORBOL ESTERS

1.4.1 Structure of PKC isozymes

Phorbol esters are tumour promoting agents, the first identified effectors of the PKC family of serine/threonine kinases (Buchner 1995). The natural ligand for PKC activation is diacylglycerol (DAG), a lipid second messenger generated by PLC directly or by PLD/phosphatidic acid (PA) pathway indirectly (Brose, Rosenmund et al., 2002). Phorbol esters mimic DAG binding and DAG or phorbol esters are required for the reversible recruiting of PKC to membranes.

PKC comprises of at least 10 structurally related phospholipid-dependent protein kinases. PKC isozymes are grouped into three subclasses according to their regulatory properties (Figure 1.5 Structures of PKC, PKC related kinases and novel ‘nonkinase’ phorbol ester receptors). (1) The ‘conventional’ or ‘classical’ PKCs (cPKCs) include PKC α,β1,β11 and γ. These isoforms can be activated by calcium and/ or by DAG, phorbol esters and insulin. (2) The ‘novel’ PKCs (nPKCs) δ, ε, θ and η can also be activated by DAG, phorbol esters and insulin but are calcium independent. (3) Finally, the ‘atypical’ PKCs which include PKCζ and PKCt (mouse homologue is called PKCλ), are not activated by calcium, DAG and phorbol esters but are activated by insulin (Mellor and Parker 1998). A related enzyme, PKCμ or PKD shows multiple unique features that makes it a distant relative of the PKC isozymes. Each PKC isozyme is a product of a separate gene except PKC β1 and β11, which are, spliced variants of the same gene (Figure 1.5 Structures of PKC, PKC related kinases and novel ‘nonkinase’ phorbol ester receptors).
Each PKC isozyme comprises of a single polypeptide chain having two structurally well defined domains; the carboxyl-terminal catalytic domain and the amino-terminal regulatory domain. The carboxyl-terminal kinase domain contains motifs involved in ATP and substrate binding. The regulatory domain comprises of motifs involved in the binding of phospholipid co-factors and calcium and is involved in protein-protein interactions that regulate PKC localisation and activity. Both the N-terminal and C-terminal domains are connected by a hinge region that is highly sensitive to proteolytic cleavage by cellular proteases.

The enzymes have regions which are highly conserved between different PKC isozymes (regions C1 to C4) and variable regions (regions V1 to V5). The C1 domain, a motif of 50 or 51 amino acids found in the N-terminal regulatory region of PKC, is required for phorbol ester/DAG binding. This was established by deletional and site-directed mutagenesis studies (Ono, Fujii et al., 1989). This region comprises of the cysteine-rich domains, which are involved in binding of the second messenger DAG and phorbol esters in cPKCs and nPKCs. The cPKCs and nPKCs have two copies of these motifs in tandem, whereas aPKCs and other proteins such as α and β chimaerins, Unc-13, Munc 13 isoforms, Ras-GRP and Raf have only one copy (Kazanietz 2002; Kazanietz 2000; Ron and Kazanietz 1999) (see section 1.4.3). The cPKCs and nPKCs posses a C2 region that in cPKCs is involved in calcium binding but in nPKCs is unable to bind calcium.
1.4.2 Functions of PKC

Many end-point responses stimulated by both phorbol 12-myristate 13-acetate and growth factors, such as insulin, overlap to some extent: for example, gene expression (Buchner 1995), membrane trafficking and membrane ruffling. The involvement of PKC isoforms in insulin-induced and phorbol ester-induced glucose transport were studied by expressing wild type and mutant PKC isoforms; conventional PKCα, novel PKCδ, and atypical PKC isoforms of PKCλ and PKCζ; in 3T3-L1 adipocytes using an adenovirus-mediated gene transduction system (Tsuru, Katagiri et al., 2002). Over-expression of each wild-type PKC isoform increased PKC activity in 3T3-L1 adipocytes. Expression of the wild-type PKCα, or PKCδ resulted in significant increases in glucose transport activity in the basal and PMA-stimulated states. Dominant-negative PKCα expression, inhibited the PMA activation of PKCα, decreased the PMA-stimulated glucose transport. In conclusion such data demonstrate that conventional and novel PKC isoforms are involved in PMA-stimulated glucose transport whereas atypical PKC enzymes do not play a major role in insulin-stimulated glucose transport in 3T3-L1 adipocytes (Tsuru, Katagiri et al., 2002). In comparison to these studies recent reports have shown that atypical PKCs are in fact required for insulin-stimulated glucose transport (Bandyopadhyay, Standaert et al., 2004).

Phorbol ester or insulin stimulation of HEK 293 cells leads to an increase in phosphorylation of 4E-BP1 and p70 S6 kinase, eIF4F assembly, and protein synthesis; all of these responses are blocked by rapamycin a specific inhibitor of mTOR (Herbert, Kilhmas et al., 2000). PI3K and PKB are both activated by insulin but not by phorbol esters. However, phorbol esters can induce eIF4F assembly, protein synthesis, and the
phosphorylation of p70 S6 kinase and 4E-BP1 independently of both PI3K and PKB. Reports show that Erk activation is important in phorbol ester stimulation of eIF4F assembly, and phosphorylation of p70 S6 kinase, and 4E-BP1. Increased eIF4E phosphorylation alone does not affect the assembly of the eIF4F complex or general protein synthesis (Herbert, Kilhmas et al., 2000).

Phorbol esters can also influence endocytosis. Endocytosis is a process in which a substance gains entry into a cell without passing through the cell membrane and is an essential process for cell function. There are two different types of endocytosis: receptor-mediated endocytosis and fluid-phase endocytosis. Reports have been made that phorbol esters can affect endocytosis. In human neutrophils it has been shown that phorbol esters or diacylglycerol can stimulate endocytosis (Keller 1990).

1.4.3 Novel ‘nonkinase’ phorbol ester/ DAG receptors

For many years it was thought that the only receptors for phorbol esters and DAG were cPKCs and nPKCs. Not all the phorbol ester responses are mediated through PKCs however. Additional phorbol ester receptors have been identified: (1) mammalian α and β chimaerins, (2) Ras-GRP, and (3) Caenorhabditis elegans Unc-13 (Kazanietz 2002; Kazanietz 2000; Ron and Kazanietz 1999) (Figure 1.5 Structures of PKC, PKC related kinases and novel ‘nonkinase’ phorbol ester receptors). Each of these receptors comprise of a single copy of the cysteine-rich domain suggesting that only one copy of the motif is required for high affinity binding of phorbol esters and DAG.
1.4.3.1 α and β chimaerins

Evidence of the first non-kinase phorbol ester receptor came from the cloning of a 38kDa protein called α1-chimaerin. The protein is highly expressed in brain and resembles a chimera of the regulatory domain of PKC and BCR (breakpoint cluster region protein) which forms part of the BCR/Abl chimaera that characterises the Philadelphia chromosome in chronic myelogenous leukaemia. Deletion of the cysteine-rich domain of α1-chimaerin leads to the complete loss of phorbol ester binding (Ahmed, Kozma et al., 1990). The chimaerin family has expanded with the cloning of novel isoforms α2-, β1-, and β2-chimaerins. The chimaerins do not have a kinase domain, but rather have a carboxyl-terminal GAP (GTPase-activating protein) domain. Both α and β chimaerins accelerate in vitro the hydrolysis of GTP bound to Rac (member of the Rho family of small GTP binding proteins) with little or no effect on Cdc42 and Rho (Ahmed, Lee et al., 1993).

1.4.3.2 Unc-13 and related proteins

The Unc-13 gene from Caenorhabditis elegans encodes a large protein 1734 amino acids. Mutations in the Unc-13 gene cause a severe phenotype characterised by its abnormal neuronal connections and impairment in cholinergic neurotransmission in the nematode. Cloning of this gene has shown a central domain with sequence similarity to the regulatory domain of PKC, which includes C1 and C2-related domains. Unc-13 protein comprises of a single cysteine-rich domain with high sequence homology to those found in PKCs and chimaerins (Maruyama and Brenner 1991). There are three Unc-13
mammalian homologues: Munc 13-1, Munc13-2 and Munc13-3; however these proteins have not been examined for phorbol ester/DAG binding (Brose, Hofmann et al., 1995). Structural similarities between cysteine-rich domains of Munc13 and other receptors suggest that they are high affinity receptors for phorbol esters. The demonstration that Munc13 interacts with proteins of the exocytotic machinery, including Doc2, syntaxin, synaptobrevin and synaptotagmin suggests a novel role of these proteins as scaffold structures (Orita, Naito et al., 1997).

1.4.3.3 RasGRP (guanyl nucleotide releasing protein)

RasGRP has been recently been isolated through a cloning approach to identify proteins that enhance Ras signalling (Ebinu, Bottorff et al., 1998). RasGRP is highly expressed in brain, thymus, bone marrow and spleen. Sequence analysis of RasGRP shows a single cysteine-rich domain at the carboxyl-terminal region that resembles that found in PKC isozymes. Phorbol ester binding studies has confirmed that RasGRP is indeed a phorbol ester receptor (Ebinu, Bottorff et al., 1998). RasGRP comprises of a catalytic region, the CDC25 box, named for the prototypic Ras activator from *Saccharomyces cerevisiae*, and a Ras exchange motif (REM), which is conserved among the guanyl nucleotide-releasing factors interacting with members of the Ras family. The CDC25 box has approximately 50% similarity with the Ras activators SOS1 and RasGRF1. The phorbol ester-binding site is involved in recruitment of RasGRP to the plasma membrane where it can activate Ras. Recruitment of RasGRP to the plasma membrane by phorbol esters results in the activation of downstream effectors of Ras for example, MAPK. Experiments using a mutated RasGRP lacking the cysteine rich domain
failed to translocate and activate MAPK after PMA treatment (Ebinu, Bottorff et al., 1998; Tognon, Kirk et al., 1998).

1.5 SIGNAL TRANSDUCTION AND THE MAPK SUPERFAMILY

The MAPK superfamily is made up of three main and distinct signalling pathways: ERKs, JNK or stress-activated protein kinases (SAPK), and the p38 family of kinases. The stimuli and nuclear substrates of the MAPKs mentioned above are summarised in Table 1.1.

1.5.1 ERK

ERK responds primarily to growth factors and mitogens and stimulates transcriptional responses in the nucleus. MAPK/ERK kinase (MEK) 1 and MEK2 activate ERK1 and ERK2 at a Thr-Glu-Tyr motif. The MEKs in turn are activated by c-Raf, the MAPKKK of this signalling pathway, that are in turn regulated by growth factor receptors and tyrosine kinases activating through Ras (Kolch 2000). ERKs are responsible for the phosphorylation of multiple substrates; these include transcription activators such as p90 RSK S6 kinase, MAPK-activated protein kinase-1, MAPKAP-K1, phospholipase A2 and MSK, transcription factors such as STAT (signal transducers and activators of transcription) proteins (Table 1.1). Activation of the ERK pathway mediates cell division, migration and survival.

Recent reports have shown that the cytokine TNFα induces expression of Foxc2 (winged helix/fork head gene) via a class I PI3K and ERK 1/2 dependent mechanism in
adipocytes (Gronning, Cedergberg et al., 2002), suggesting that PI3K is upstream of MAPK. A review of the literature also reveals that activation of ERK is not required for insulin stimulation of GLUT4 translocation in muscle (Berti and Gammeltoft 1999; Chen, Zheng et al., 1995), but it is required for the leptin and AICAR stimulated translocation of GLUT4 translocation in muscle (Berti and Gammeltoft 1999; Chen, Zheng et al., 1995).

1.5.1.1 JNK/SAPK family of kinases

The JNK/SAPK pathway is homologous to the MAPK and is also activated largely by distinct stimuli (Kyriakis and Avruch 1990). A variety of extracellular stimuli can activate the JNK/SAPK pathway including inflammatory cytokines, UV light, inhibitors of protein synthesis and osmotic stress (Table 1.1) (Kyriakis and Avruch 1990; Kyriakis, Banerjee et al., 1994). The JNKs are activated by dual phosphorylation at a Thr-Pro-Tyr motif by JNKK1 and JNKK2, also known as MAPK kinase 4 (MKK4) and MKK7. The JNK's can also be activated by insulin (D-Mouthon, Eggelpoel et al., 1998).

1.5.1.2 p38 family of kinases

Like the SAPK/JNK pathway, p38 kinases are also activated by environmental stresses such as heat, osmotic and oxidative stresses, ionising radiation, growth factors as well as cytokines (Table 1.1) (Raingeaud, Gupta et al., 1995). The upstream kinases acting on p38 include MKKs 3 and 6. Activated p38 kinase has been shown to phosphorylate and activate MAPKAP kinase 2 and transcription factors ATF-2 (Raingeaud, Gupta et al., 1995), Max and MEF2.
Table 1.1 Stimuli and nuclear substrates of MAPK

<table>
<thead>
<tr>
<th>MAP kinase</th>
<th>Stimuli</th>
<th>Nuclear substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERK1/2</td>
<td>Growth factors</td>
<td>Elk1, c-Myc, SAPs, c-Jun, RSKs, MSK, Mnks</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
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<tr>
<td></td>
<td>Hormones</td>
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<tr>
<td></td>
<td>Cytokines</td>
<td></td>
</tr>
<tr>
<td>p38 isoforms</td>
<td>Hormones</td>
<td>ATF-2, MEF2, SAPs, STAT3, MAPAPs, MSK, Mnks</td>
</tr>
<tr>
<td></td>
<td>Cytokines</td>
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<td></td>
<td>Osmotic stress</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Heat shock</td>
<td></td>
</tr>
<tr>
<td>JNK isoforms</td>
<td>Hormones</td>
<td>c-Jun, Elk-1, STAT3</td>
</tr>
<tr>
<td></td>
<td>Cytokines</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Osmotic stress</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inhibitors of DNA and protein synthesis</td>
<td></td>
</tr>
<tr>
<td>ERK5</td>
<td>Growth factors</td>
<td>MEF2, RSKs</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hormones</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Osmotic stress</td>
<td></td>
</tr>
<tr>
<td>ERK3 and ERK7</td>
<td>None identified</td>
<td>None identified</td>
</tr>
</tbody>
</table>
1.6 INHIBITORS FOR ELUCIDATING MECHANISMS OF ACTION OF PI3K

1.6.1 PI3K related inhibitors and their mode of action

To elucidate the physiological processes in which PI3K is involved, inhibitor studies are often used. Wortmannin and LY294002 are structurally different inhibitors that at low doses are specific inhibitors of PI3K (Davies, Reddy et al., 2000; Stein and Waterfield 2000).

Wortmannin is a potent inhibitor of PI3K with a IC$_{50}$ in the low nanomolar range (1-10nM) for the PI3K family members, with the exception of PI3K-C2α, which has an IC$_{50}$ value in the order of 500nM (Domin, Pages et al., 1997; Virbasius, Guilherme et al., 1996). Wortmannin will also inhibit PI 4-kinase, MLCK (Nakanishi, Kakia et al., 1992) and PLD at a higher concentration. Wortmannin is mostly an irreversible inhibitor that reacts covalently with lysine residues in the ATP binding site of PI3Ks. However, wortmannin is unstable in aqueous solution therefore this led to the use of a more stable compound called LY294002. LY294002 is a less potent inhibitor of PI3K and has an IC$_{50}$ of 1μM for PI3K. Recent studies have shown that LY294002 can inhibit casein kinase 2 (CK2) with a similar potency to PI3K, whereas CK2 was found to be resistant to wortmannin (Davies, Reddy et al., 2000). These results might have implications for the interpretation of results and both inhibitors should be used with caution. LY294002 is a reversible inhibitor competitive for the ATP binding site of PI3K (Vlahos, Matter et al., 1994). The main properties of the PI3K inhibitors wortmannin and LY294002 are summarised in Table 1.2. Several other molecules are currently under development as isoform-specific PI3K inhibitors but they are subjects of unpublished patent applications.
### Table 1.2 Comparison of Wortmannin and LY294002 characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Wortmannin</th>
<th>LY294002</th>
</tr>
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<tbody>
<tr>
<td>Site of action</td>
<td>ATP-binding site</td>
<td>ATP-binding site</td>
</tr>
<tr>
<td>Mode of action</td>
<td>Irreversible inhibitor</td>
<td>Reversible inhibitor</td>
</tr>
<tr>
<td></td>
<td>Alkylates a Lys in the ATP binding site</td>
<td></td>
</tr>
<tr>
<td>Sensitivity of other PI3Ks</td>
<td>All 2-5nM except PI3K-C2α (IC₅₀ approx 500nM)</td>
<td>All 0.5-1.5µM except PI3K-C2α (IC₅₀ approx 20µM)</td>
</tr>
<tr>
<td>Stability in aqueous solution</td>
<td>Poor</td>
<td>Good</td>
</tr>
<tr>
<td>Cellular effects</td>
<td>Growth inhibition (20-50nM)</td>
<td>Growth inhibition (5-20µM)</td>
</tr>
<tr>
<td></td>
<td>Apoptosis (higher concentration)</td>
<td>Apoptosis (higher concentration)</td>
</tr>
</tbody>
</table>
1.6.2 Other inhibitors of PI3K

As well as using the well known PI3K inhibitors wortmannin and LY294002, naturally occurring compounds such as the methylxanthines, caffeine and theophylline can also inhibit members of the PI3K related family of kinases ATM and ATR (Blasina, Price et al., 1999; Sarkaria, Busby et al., 1999). Caffeine and theophylline are found in human circulation as a result of dietary intake or pharmacological use. These compounds seem to effect stimulation of muscle contraction, have anti-inflammatory and immunomodulatory effects, alterations in glucose metabolism, attenuation of the antilipolytic effect off insulin, and induction of apoptosis. They have identifiable actions in vitro that include phosphodieserase inhibition; this enzyme is responsible for breaking down cAMP, thus leading to an increase in cellular cAMP levels. These levels of cAMP then antagonise with adenosine receptors and cause an increase of cytosolic calcium.

During our studies we found that caffeine and theophylline can be of particular use in dissecting the specific role of the lipid and protein kinase activity of PI3K especially p110δ (Foukas, Daniele et al., 2002). This finding is of physiological relevance as methylxanthines, such as caffeine and theophylline are compounds that are found in human circulation as a result of dietary intake or pharmacological use. With the work of Dr Lazaros Foukas (colleague at the time), we found that caffeine and theophylline both inhibit the intrinsic protein kinase activity of the class IA PI3Ks and DNA-dependent protein kinase; however, a much lower potency of inhibition was observed compared to that for the lipid kinase activity. We found that caffeine and theophylline both inhibited class I and class II PI3K lipid kinase activity with a similar potency. The inhibitory
effects of these methylxanthines on PI3K were further confirmed by experiments using intact cells or animal tissues where these compounds inhibited insulin-stimulated PKB activation, and glucose transport, since both of these processes are strictly dependent on PI3K. Overall our results show that inhibition of PI3K and p110δ in particular, are likely to explain some of the physiological and pharmacological properties of caffeine and theophylline. Our results have implications for future studies of PI3K, as experiments are not just limited to the use of wortmannin and LY294002, but can employ these novel inhibitors too.
1.6.3 PKC and ERK related inhibitors and their mode of action

The two most commonly used cell permeable inhibitors of MEK1/2 are PD98059 and U0126 (Davies, Reddy et al., 2000; Dudley, Pang et al., 1995; Favata, Horiuchi et al., 1998). Most kinase inhibitors compete with ATP for binding to the active site of a kinase, however both these inhibitors work by different mechanism. PD98059 has been shown to be a highly selective inhibitor of MEK1 activation, and the MAPK cascade (Alessi, Cuenda et al., 1995; Dudley, Pang et al., 1995; Pang, Sawada et al., 1995). PD98059 binds to the inactive forms of MEK1 and prevents activation by upstream activators such as c-Raf (Pang, Sawada et al., 1995). PD98059 inhibits activation of MEK1 and MEK2 with IC50 values of 4μM and 50μM respectively (Alessi, Cuenda et al., 1995; Dudley, Pang et al., 1995; Pang, Sawada et al., 1995). U0126 is a specific inhibitor of MEK1 and MEK2 (Favata, Horiuchi et al., 1998). Both inhibitors are non-competitive with respect to the MEK substrates ATP and ERK (Favata, Horiuchi et al., 1998). The binding sites for PD98059 and U0126 on MEK1 appear to overlap, and their mechanisms of action differ. U0126 is a direct inhibitor of MEK kinase activity, whereas PD98059 inhibits MEK activation. Here in this study we have used another inhibitor of MEK, the ste-MPKKKPTPIQLNP MEK derived peptide which blocks activation of ERK, and which has been modified to allow cell permeability (Kelemen, Hsiao et al., 2002). Other inhibitors we used for these studies include the inhibitor of the JNK/SAPK pathway SP600125. SP600125 is an athrapyrazolone inhibitor of JNK (Bennett, Sasaki et al., 2001). SP600125 is a reversible ATP competitive inhibitor with more than 20-fold selectivity versus a range of kinases and enzymes.
We also used an inhibitor of the PKC pathway called Bisindolylmaeimide I (BIM-1). BIM-1 is a highly selective cell permeable PKC inhibitor which is structurally similar to staurosporine (Toullec, Paianetti et al., 1991). This inhibitor acts as a competitive inhibitor for the ATP binding site of PKC showing high selectivity for PKCα, βI, βII, γ, θ and ε isozymes. All these inhibitors were used to elucidate the signalling pathways by which class II PI3K is regulated.
Figure 1.1 Chemical structure of PtdIns
(a) The myo-D-enantiomer of inositol is shown, in which the 2-hydroxyl is axial and the other hydroxyls are equatorial. (b) List of PI known to exist in mammalian cells. PtdIns-4-P and PtdIns-4,5-P₂ represent approximately 60% and 30%, respectively, of the total PIs in cells (adopted from Fruman and Cantley 1998).
Figure 1.2 Synthetic pathways for PIs.

This figure is a simplified version taken from the original adopted from Kanaho & Suzuki, 2002). To date, eight PIs have been identified and they are indicated in blue. The PI kinases and reactions catalyzed by them are shown in red and black.
Figure 1.3 Structures of different classes of PI3K.
Class IA and B, class II, class III PI3K and mTOR (sometimes called class IV) indicating all domains within the structure (adopted from Vanhaesebroeck et al., 2001)
Figure 1.4 Insulin stimulation of PI3K
The schematic diagram shows how PI3K is stimulated after insulin binds to its receptor and causes autophosphorylation on tyrosine residues hence tyrosine phosphorylation of insulin receptor substrates (IRS) with the phosphotyrosine binding (PTB) domain. This leads to association of the regulatory subunit of PI3K p85 through its SRC homology domains (SH2) with IRS’s. In turn this results in recruitment of PI3K to the plasma membrane where it phosphorylates PtdIns-4,5-P₂ on the D-3 position to produce PtdIns-3,4,5-P₃.
Chapter 1

PKC

PKC regulatory domain

PKC catalytic domain

V1 V2 V3 V4 V5

C1

cPKCs (PKC α, βI, βII, γ)
nPKCs (PKC δ, ε, η, θ)
aPKCs (PKC ζ, τ, λ)

PKC-RELATED KINASES

PKC μ / PKD

PRKs

NON-KINASE PHORBOL ESTER RECEPTORS

α1(n)-and β1-chimaerin

α2(n)-and β2-chimaerin

Munc13-1

Munc13-2/3, Unc13

Ras-GRP

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Figure 1.5 Structure of PKC, PKC related kinases, and novel “nonkinase” phorbol ester receptors.

The different isoforms and related proteins are shown; cPKC are the conventional or classical PKCs, nPKC are the novel PKCs and aPKC are the atypical PKCs.

The domains are as follows: pseudosubstrate; cysteine-rich domain; C2 domain; C3 domain; kinase domain; transmembrane domain; PH domain; rho binding domain; Rac-GTPase-activating protein domain; SH2 domain; Ras exchange motif; CDC25, region with homology to *Saccharomyces cerevisiae* CDC25 and guanine nucleotide exchange factor domain of Sos; proline-rich domain; EF hand motif (adopted from Ron & Kazanietz, 1999).
1.7 AIMS OF THE STUDY

The class II PI3Ks have not been widely studied and the available evidence on their functional role in the cell is conflicting. In particular the mechanism by which class II PI3K is regulated remains unknown. Class II PI3K is activated by a range of growth factors (Brown, Domin et al., 1999; Urso, Brown et al., 1999), chemokines (Turner, Domin et al., 1998) and integrins (Zhang, Banfic et al., 1998; Paulhe, Perret et al., 2002). In the current study we have investigated how different stimuli regulate class II PI3K and the mechanism by which this regulation occurs.

To begin such studies we investigated whether key-signalling pathways such as PKC and ERK could be involved in either increases in phosphorylation or activation of class II PI3K. As a result we embarked upon a project involving a range of experimental approaches, aiming to resolve the issue of the regulation of PI3K-C2α.

The aims of this project can be summarised as follows:

- To create antisera directed against the N-terminal domain of the human sequence of PI3K-C2α for experimental analysis.
- Testing the effects of growth factors such as insulin, phorbol esters such as PMA, cytokines such as leptin, and TNFα, on the activation, phosphorylation of PI3K-C2α and the association of PI3K-C2α with tyrosine phosphorylated proteins.
- To use inhibitors of candidate signalling cascades, such as PKC and ERK, to investigate the mechanisms by which insulin, PMA, TNFα and leptin regulate activation and phosphorylation of PI3K-C2α in cell line models.
• To test the effects of phorbol esters on the production of PtdIns-3-P in cell line models and to use inhibitors of candidate pathways such ERK to identify the mechanism by which PMA induces production of this lipid.

• To test the effects of insulin and adrenaline on the activation and recruitment of class I and class II PI3K (PI3K-C2α) in rat muscle.
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2. MATERIALS AND METHODS

2.1 MATERIALS

Tissue culture media, phosphate-buffered saline (PBS), trypsin and fetal calf serum were obtained from Sigma. Oligofectamine and lipofectamine were from Invitrogen Life Technologies. Chemicals were from Sigma, unless otherwise stated. Polyclonal PI3K-C2α antibodies were generated as described (See section 2.2.4.1). ERK1/2, MEK1/2, PKCα/βII and SAPK/JNK antibodies were from Cell Signalling. Phosphotyrosine antibodies (PY-99), FAK (C-20) and cbl-b (H-454) were from Santa Cruz. p130cas antibody was from BD Biosciences. Agarose-conjugated protein A and agarose conjugated anti-mouse IgG was from Sigma. [γ³²P]ATP (specific activity: 10mCi/ml) was from NEN. PIs were obtained from Sigma. Phorbol-12-Myrisate-13-Acetate (PMA), 4α-Phorbol, Bisindolymaleimide I (BIM-1), rapamycin, Ste-MEK113 peptide, H-89 and Wortmannin were from Calbiochem. PD98059 was from Cell signalling. U0126 and insulin was from Sigma. SP600125 was from Tocris. cAMP analogue (8'-CPT'-'cAMP) was from Sigma. Activated recombinant ERK1 was from Upstate Biotechnology. Recombinant murine leptin was from Biogenesis. Recombinant murine TNFα was from R&D systems. Constitutively active PKCα plasmid DNA was a kind gift of the Parker research laboratory. The sources of other reagents were stated in the text as necessary.
2.2 METHODS

2.2.1 Cell Culture

All procedures were carried out in a laminar flow hood at 37°C with 5% CO\textsubscript{2} using aseptical technique. All surfaces were disinfected with 70% ethanol prior and after any manipulation. Media for mammalian cell culture were pre-warmed to 37°C before use.

2.2.1.1 Freezing of cells

Briefly, cell lines were kept in liquid nitrogen for long-term purposes. Aliquots were removed from storage and thawed quickly at 37°C in a water bath before transfer to a tissue culture flask containing pre-warmed culture medium. Following overnight incubation at 37°C in a humidified incubator with 5% CO\textsubscript{2} for mammalian cells, medium was changed to remove traces of the cryopreservation medium. Cells were removed from the surface of the dish by harvesting cells using trypsin, and the cell suspension was transferred into a 10ml sterile falcon tube. The cells were pelleted by centrifugation at 150xg for 7 minutes at 4°C. The supernatant was aspirated and the cell pellet resuspended in ice cold freezing medium (culture media containing 10% (v/v) fetal calf serum and 10%(v/v) DMSO) to give a cell density of 1-2x10\textsuperscript{6} cells/ml. 1ml aliquots of the suspension cells were transferred to sterile cryovials, which were frozen slowly in a polystyrene box in a -80°C freezer overnight. The following day, cells were transferred to liquid nitrogen for long-term storage.
2.2.1.2 Maintenance of cells

2.2.1.3 Maintenance of HEK 293, L6 myoblasts and CHO-IR cells

Human embryonic kidney (HEK 293) and L6 myoblast cells were cultured in Dulbecco Modified Eagle Essential Medium (DMEM) supplemented with 10% (v/v) fetal calf serum and 1% (w/v) antibiotic-antimycotic. Chinese Hamster Ovary cells over-expressing the insulin receptor (CHO-IR) were cultured in nutrient HAMS F-12 media supplemented with 10% (v/v) fetal calf serum and 1% (w/v) antibiotic-antimycotic. Stock flasks of CHO-IR also contained 250μg/ml of G418 antibiotic. When all cells became confluent cell culture medium was removed and the monolayers were briefly washed with PBS (without calcium or magnesium) before addition of Trypsin/EDTA (1ml) per 75cm² flask (TPP) for 5min at 37°C. The flasks were tapped lightly to dislodge the cells, the appropriate amount of media was added to re-suspend the cells, and plated at a density of approximately 60% of confluence.

2.2.1.4 Maintenance of 3T3-L1 fibroblast cells

3T3-L1 fibroblasts were cultured in DMEM containing 4.5g/l glucose supplemented with 10% (v/v) New born calf serum and 1% (w/v) antibiotic-antimycotic. Stock flasks of fibroblasts were trypsinised at sub-confluency to avoid differentiation. Cell culture medium was removed and fibroblasts were briefly washed with PBS (without calcium or magnesium) before addition of Trypsin/EDTA (1ml) per 75cm² flask (TPP) for 5min at 37°C. The flasks were then tapped lightly to dislodge cells from the surface, the
appropriate amount of media was then added to re-suspend cells and plated at a density of approximately 60% of confluence.

2.2.1.5 Differentiation of fibroblasts to adipocytes

Fibroblasts were differentiated two days after confluence had been reached, using Frost and Lane method, (Frost and Lane, 1985). Cells were incubated for two days in DMEM (4.5g/l glucose) containing 10% (v/v) fetal calf serum, 1% (w/v) antibiotic-antimycotic, 1µg/ml insulin, 0.25µm Dexamethazone and 0.5mM IBMX (3-isobutyl-1-methyl-xanthine). At day 2 of differentiation the medium was then changed to DMEM containing 10% (v/v) fetal calf serum, 1% (w/v) antibiotic-antimycotic, and 1µg/ml insulin. From day 4, cells were fed every 48 hours with DMEM containing 10% (v/v) fetal calf serum and 1% (w/v) antibiotic-antimycotic only. Adipocytes were then used after day 7 and before day 12.

2.2.1.6 Maintenance of murine macrophage cell line (J774.2)

Murine macrophage cell line were grown in RPMI medium 2g glucose/l supplemented with 10% (v/v) fetal calf serum (heat inactivated for 30mins at 50°C) and 1% (w/v) antibiotic-antimycotic per 10cm dish (NUNC, Gibco) or 75cm² flasks (Falcon Marathon labs). The medium was removed and replaced with fresh media every 48hrs. To passage the cells the medium was removed and cells were scraped from the bottom of the flask. Cells were resuspended in an appropriate amount of media and plated at a density of approximately 60% of confluence.


2.2.2 Transfection studies

2.2.2.1 Maxiprep plasmid purification for transfection studies

To produce plasmid DNA for transfections the following procedures were carried out. A 5ml starter culture containing the desired DNA transformed into *E.coli* was grown in Luria Broth (LB) (see section 2.2.4.2). Inoculation with a single colony isolated from a freshly streaked agar plate containing 100μg/ml ampicillin (see section 2.2.4.2) was incubated with shaking for 8h at 37°C. The starter culture was then used to inoculate a 100ml culture by diluting 1:500. After incubating overnight at 37°C with shaking, cells were harvested by centrifugation at 4000rpm for 15min at 4°C. Pelleted cells were further processed using a QIAfilter™ Plasmid Maxi Kit (Qiagen) according to the manufacturers instructions. Purified plasmid DNA was now suitable for transfection experiments.

2.2.2.2 Determination of DNA concentration

DNA concentration was determined spectrophotometrically by measuring Optical Density (OD) at 260nm. Concentration was then calculated from the equation:

\[ \text{OD}_{260} = 1 = 50 \mu g/ml \text{ double stranded DNA} \]
2.2.2.3 Transfection of CHO-IR cells

CHO-IR cells were transfected using LipofectAMINE (Invitrogen). Briefly, confluent cells were trypsinized, replated at a dilution of 1:10 in 6 well dishes, and grown overnight. The next day the monolayers were rinsed with PBS and plain F12 medium was added. Each of plasmid DNA (2μg per well in 6well dish) and LipofectAMINE reagent (5μl) were diluted with OPTIMEM I Reduced Serum Medium to 800μl final volume. The media containing DNA and LipofectAMINE were mixed together and incubated for 45min at room temperature. The mix was added to the monolayer and the cells were incubated with transfection mixture for 6 hours, before the medium was replaced with HAMS-F12 containing 10% (v/v) fetal calf serum and 1% (w/v) antibiotic-antimycotic. Protein expression from the transfected plasmids was found to be optimal 24h post-transfection.

2.2.2.4 Stimulation of cells

CHO-IR, HEK293 and L6 cells were serum starved for 16hrs in Nutrient HAMS F-12 and DMEM prior to stimulation. 3T3-L1 adipocytes were serum starved for 1hr after differentiation (after day 7 before day 12) in DMEM low glucose with 1% (w/v) antibiotic-antimycotic and Bovine serum albumin (BSA) (5.5mg/ml) prior to stimulation. J774.2 macrophages were serum starved for 24hrs in RPMI media supplemented with 0.1% (w/v) BSA (fatty acid free).

Cells were treated with insulin (100nM) as we have previously shown (Brown, Domin et al., 1999), PMA (1μM) as we have previously shown (Nave, Siddle et al., 1996), MEK
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inhibitor PD98059 (50μM) (Alessi, Cuenda et al., 1995), MEK inhibitor U0126 (10μM) (Favata, Horiuchi et al., 1998), cell permeable Ste-MEK113 peptide (50μM) (Kelemen, Hsiao et al., 2002), JNK inhibitor SP600125 (25μM) (Davies, Reddy et al., 2000), PI3K inhibitor wortmannin (100nM) (Domin, Pages et al., 1997; Brown, Domin et al., 1999), PKC inhibitor BIM-1 (5μM) (Toullec, Paianetti et al., 1991), mTOR inhibitor Rapamycin (50nM) (Nave, Ouwens et al., 1999), PKA inhibitor H-89 (0.1μM) (Davies, Reddy et al., 2000), cAMP analogue (8’-CPT’-cAMP) (100μM), Leptin (2nM) (O’Rourke, Yeamen et al., 2001) or TNFα (25ng/ml) (Gronning, Cedergberg et al., 2002) in the appropriate pre-warmed medium (37°C), in a laminar flow hood providing a sterile environment.

2.2.3 Muscle preparation

2.2.3.1 Muscle preparation and incubation with agonists

Muscles were prepared from male Wistar rats and incubations and contractions were performed as previously described (Aslesen and Jensen 1998). Briefly dissected muscles were suspended on a contraction apparatus between 2 platinum electrodes at their approximate resting length. The muscles were preincubated in oxygenated (95%O₂ /5%CO₂) Krebs-Henseleit buffer containing 5.5mM glucose, 2mM sodium pyruvate, 5M HEPES, 0.1% (w/v) BSA for 30min at 30°C. Muscles were stimulated with insulin (10mU/ml) and/or adrenaline (10μM) for 30min unless otherwise stated. The above procedures were carried out by our collaborators, Dr Jorgen Jensen and his research laboratory.
2.2.3.2 Preparation of muscle extracts for signalling studies

After incubations, muscles were frozen in liquid nitrogen and stored at -80°C. Subsequent steps were performed at 4°C. Samples were homogenised in ice cold buffer (1% (v/v) Triton-X-100, 50mM HEPES, 150mM NaCl, 10mM Na₃P₂O₇, 10mM EDTA, 0.1% (w/v) SDS, 2μg/ml aprotinin, 1mM pepstatin and 1μg/ml leupeptin, 2.5mM benzamidine and 0.5mM PMSF) using a Glass homogenizer (Wheaton). Homogenates were rotated for 1hr and insoluble matter removed by centrifugation at 13000xg for 10min.

2.2.3.3 Determination of protein concentration

Protein concentration was determined using the Pierce BSA protein assay reagent (Protein assay reagent A, BCA protein assay reagent B and Albumin standard 2.0mg/ml in 0.9% (w/v) aq NaCl solution containing sodium azide).

2.2.4 Immunological Methods

2.2.4.1 Generation of PI3K-C2α antibodies

Polyclonal antibodies against the N-terminal domain of PI3K-C2α were generated by immunising rabbits with GST expressed fusion protein in vector pGEX 4T-1 corresponding to amino acids 9-366 of the human sequence of PI3K-C2α. To do this briefly, plasmid DNA provided was transformed into *E.coli* cells then expressed and
purified. This expressed protein was then used to immunize two rabbits to generate antisera. Antisera were then affinity purified and used for research purposes.

2.2.4.2 Transformation of competent *E.coli*

Plasmid DNA (1μl) was added to 100μl of freshly thawed competent cells (XL Blue1), mixed, and incubated on ice for 10mins. Cells were heat shocked at 42°C for 90secs, kept on ice for 90secs and then allowed to recover in 1ml LB for 1hr with shaking at 37°C. The culture was centrifuged (UFO 10,000g for 30secs) and the supernatant removed. The pellet was re-suspended in 100μl LB and then spread over a LB agar plate containing 100μg/ml ampicillin. The plates were incubated overnight at 37°C.

2.2.4.3 Expression and purification of GST fusion proteins

A single colony was inoculated into 10ml LB containing 100μg/ml ampicillin and kept shaking overnight at 37°C. The next day the culture was used to inoculate a 1000ml culture in LB medium supplemented with 100μg/ml ampicillin. The culture was grown at 37°C with shaking and OD readings were recorded at 600nm. When OD readings reached exponential phase (approximately 0.6) the culture were induced by addition of 1mM IPTG. Following a further incubation of 3 hours, the bacterial cells were pelleted at (10,000rpm; 4°C for 10mins). Pelleted cells were resuspended in 10 volumes (ml/g cell pellet) ice cold buffer PBS + 1% (v/v) Triton X-100, protease inhibitors aprotinin (2μg/ml), leupeptin (1μg/ml), pepstatin (1mM), PMSF (0.2mM), EDTA (2mM), DTT
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(1mM) and Benzamidine (10mM). Samples were then briefly sonicated on ice by applying six rounds of 10s pulses with intervals in between. The lysate was then centrifuged at 10000g at 4°C for 10min to remove cell debris. Protein was isolated from the lysate by rotating with Glutathione-Sepharose 4B (Pharmacia) at 4°C. Sepharose beads with bound GST fusion proteins were washed three times in ice cold buffer. The protein was eluted from the beads by the addition of elution buffer (10mM reduced glutathione, 50mM Tris HCl pH8). Expressed protein levels were detected by Coomassie staining (see section 2.2.4.8).

2.2.4.4 Generation of antibodies against the N-terminal domain of PI3K-C2α

Polyclonal antibodies against the N-terminal domain of PI3K-C2α (N-PI3K-C2α) were generated by immunizing rabbits with a glutathione S-transferase (GST) fusion protein corresponding to aminoacids 9-336 of the human PI3K-C2α sequence. 10mg of expressed protein measured using the Pierce BSA protein assay reagent (see section 2.2.3.3) were used to immunise two rabbits. Immunizations were carried out by Eurogentec (Belgium). Testing of small and large bleeds were carried out by blotting lysates from both immunisations (Fig 2.1).

2.2.4.5 Purification of PI3K-C2α antibodies

An affinity column was made by coupling GST-N-PI3K-C2α fusion protein to Actigel and filtered antiserum was then purified on this column. Columns were washed with PBS and immunoglobulins were eluted with 100mM glycine pH 2.5. The eluate was immediately neutralized by addition of 1M Tris, and concentrated using spin filters. The
N-PI3K-C2α purified antibodies were aliquoted in Ethylene glycol, and stored at -20°C.

The purified antibody preparation was characterised by immunoblotting (Fig 2.2).
Figure 2.1 Blotting of PI3K-C2α using small and large test bleeds.

CHO-IR cells were lysed, and 50μg of total lysate was subjected to SDS-PAGE and Western blotted with pre-immune serum and both the small and large bleed sera from the two different immunised rabbits (1=SK1493) and (2=SK1494). PI3K-C2α antisera (termed as old antibody) was used as a control for blotting and this antibody was provided by the Waterfield laboratory (Brown, Domin et al., 1999). Similar results were obtained in at least 3 separate experiments.
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Figure 2.2 Testing efficiency of newly purified SK1493 serum in immunoprecipitations and on blotting of PI3K-C2α.

CHO-IR cells were lysed, and lysates (1mg) were subject to immunoprecipitation using purified SK1493 serum. Immunoprecipitates and 50µg of total lysate were subjected to SDS-PAGE and Western blotted with purified SK1493 serum (1:500 or 1:1000 dilution). Similar results were obtained in at least 3 separate experiments.
2.2.4.6 Immunoprecipitation of proteins

After stimulation of cells (see section 2.2.2.4), cells were washed once with ice cold PBS (calcium and magnesium free) and lysed in 0.5ml lysis buffer (10mM Tris-HCl pH 7.4, 5mM EDTA pH 8, 50mM NaCl, 50mM NaF, and 1% (v/v) Triton X-100 supplemented with 2µg/ml aprotinin, 1mM Pepstatin, 1µg/ml Leupeptin, 2mM PMSF and 1mM sodium orthovanadate for 10cm² dishes or 0.3ml for 6 well dishes. Lysates were clarified from insoluble material by centrifugation at 12,000xg for 10mins at 4°C. Immunoprecipitations were performed on the Triton-soluble fraction (1mg of total protein) or 500µg of prepared muscle tissue using the antibody of interest at a dilution of 1:100. Lysates were incubated with antibody for 1hr at 4°C subjected to rotation followed by addition of protein A- or mouse IgG beads depending on the antibody used (20µl of 1:2 slurry). Immune complexes were washed three times with buffer (10mM Tris-HCl pH 7.4, 5mM EDTA pH 8, 50mM NaCl, 50mM NaF, and 1% Triton X-100 supplemented with 2µg/ml aprotinin, 1mM Pepstatin, 1µg/ml Leupeptin, 2mM PMSF and 1mM sodium orthovanadate, followed by three washes with kinase assay buffer for PI3K lipid kinase assays (See section 2.2.4.11).
2.2.4.7 Separation of proteins by SDS-polyacrylamide gel electrophoresis

To visualise proteins SDS-PAGE was performed as described by Laemli (Laemli 1970). Protein was loaded on a gel using the Bio-Rad Miniprotean III electrophoresis system or the Hoefer electrophoresis system.

Samples (either total cell lysate or immunoprecipitate) were denatured by the addition of electrophoresis sample buffer (4x concentrated: 0.5M Tris pH 6.8, 8% (w/v) SDS, 20% Glycerol, 2mM EDTA and 4% (v/v) β-mercaptoethanol) and heating at 100°C for 5min. SDS-PAGE gels consisting of 4% acrylamide stacking gel (375mM Tris HCl pH6.8, 0.1% (w/v) SDS and 4% (w/v) acrylamide) and a 8% resolving gel (125mM Tris-HCl pH 8.8, 0.1% (w/v) SDS and 8% (w/v) acrylamide) using Miniprotean system or 6% resolving gel with an 80:1 ratio of bis-acrylamide:acrylamide (1.5M Tris pH8.8, 0.4% (w/v) SDS, 80:1 ratio of acrylamide:bis-acrylamide) using the Hoefer electrophoresis system were used. Gels were polymerised with the addition of 0.05% (v/v) TEMED and 0.05% (w/v) Ammonium Persulphate (APS). Gel electrophoresis proceeded at 200V in electrode buffer (200mM Tris base, 2.5mM Glycine and 1% (w/v) SDS). Molecular weight markers (Rainbow markers Amersham) were also loaded on the gel. Following electrophoresis the gels were stained with Coomassie Blue or exposed to autoradiography or processed for western blotting.
2.2.4.8 Coomassie Blue staining of acrylamide gels

Following electrophoresis, the gel was placed in a solution of 40\% (v/v) methanol, 10\% (v/v) acetic acid, 0.3\% (w/v) Coomassie brilliant blue R250 and incubated under constant agitation for 1h. Protein bands were then visualised by washing the gel in a destaining solution (20\% (v/v) methanol, 7\% (v/v) glacial acetic acid).

2.2.4.9 Autoradiographic exposure of acrylamide gels

Following electrophoresis, the gel was submerged in fixing solution of 20\% (v/v) ethanol, 10\% (v/v) glacial acetic acid and incubated under constant agitation for 1h in order to fix proteins into the gel. The gel was then dried in vacuo and exposed to a phosphorimager screen. Images of radiolabelled protein were acquired using a Fuji FLA-2000 phosphorimager and analysed with Fuji Image Gauge software.

2.2.4.10 Western blotting and immunodetection of transferred proteins

After electrophoresis proteins were transferred to Polyvinylidene Fluoride (PVDF) membrane for immunodetection. Prior to blotting, the PVDF membrane was pre-soaked in methanol and then rinsed in water. The gel, membrane and Whatmann 3MM paper were equilibrated in transfer buffer (48mM Tris base, 39mM Glycine, 1\% (w/v) SDS and 20\% (v/v) methanol to minimise swelling for 5mins). A gel blot was constructed of 3 pieces of Whatmann paper either side of the membrane and the gel. The sandwich was immersed in a buffer-filled tank, and transfer conducted from the cathode to the anode.
Electrophoretic transfer proceeded for 1hr at 100V for small mini-gels and 2.5hrs at 65V for large gels.

Following transfer to PVDF membranes, the membranes were blocked with 5% (w/v) milk non-fat dried milk in PBS plus 0.1% (v/v) Tween (PBS-T) for 1hour. For immunoblotting using phosphospecific antibodies, BSA and Tris-buffered saline were used instead of skimmed milk and PBS throughout the procedure.

The primary antibody of choice was then added at the appropriate dilution in 10ml of 1% (w/v) dried milk powder in PBS-T and incubated on a rocking platform at room temperature for 1hr or at 4°C overnight. The membrane was then washed with PBS-T three times, for 5min each. The secondary antibody, conjugated to horseradish peroxidase (HRP) was diluted at 1:1000 in 10ml of 1% (w/v) milk in PBS-T and incubated with the membrane for 45min at room temperature. The membrane was then washed three times in PBS-T for 5min each. Antibody-antigen complexes were detected by Enhanced Chemiluminescence (Kirkegaard & Perry laboratories) according to the manufacturer’s instructions. Images were obtained by a Fuji LAS-1000 Luminescent Image Analyser and analysed with Fuji Image Gauge software.

2.2.4.11 PI3K lipid kinase assays

PI3K lipid kinase assays were performed on immunoprecipitates (see section 2.2.4.6) in a total volume of 50μl in a buffer containing 100mM HEPES pH 7.4, 200mM NaCl, 2mM DTT, 5mM MgCl₂, 100μM ATP (plus 0.1μCi of [γ-³²P]ATP/assay) using 200μg/ml PtdIns as a substrate which was resuspended in 1M Hepes. Reactions were started by adding a mix of the ATP and Mg²⁺, allowed to incubate for 20min at 25°C and terminated
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by the addition of 100μl (v/v) 0.1M HCl. 200μl chloroform: methanol (v/v) (1:1) were added and the mixture was vortexed. The phases were separated by centrifugation at 10,000xg for 2min and the aqueous phase was discarded. The remainder lower organic phase was washed with 80μl (v/v) of methanol: 1M HCl (1:1). After centrifugation the aqueous phase was discarded and the lower organic phase dried by evaporation.

Reaction products were re-suspended in 30μl (v/v) of chloroform: methanol (4:1) and spotted onto thin layer Silica Gel-60 plates (Merck). Thin layer silica gel plates were pre­treated with 1% (w/v) oxalic acid, ImM EDTA in water: methanol (v/v) (6:4) and baked at 100°C in an oven in order to minimise the binding of divalent cations to phosphorylated products, that could affect the migration of the plates. Thin Layer Chromotography (TLC) plates were developed in chloroform: methanol: 4M ammonia (v/v/v) (9:7:4) for analysis of PI3K. After completion of the separation, plates were dried and exposed to autoradiography. Images of radiolabelled lipid products were observed using a Fuji FLA-2000 phosphorimager and analysed with Fuji Image Gauge software.

2.2.4.12 Protein kinase assays

PI3K autophosphorylation assays were performed on immunoprecipitates (see section 2.2.4.6). Immunoprecipitates were washed as normal and then incubated in vitro with agent of interest. For example, recombinant active ERK (100 or 200ng), 5ng recombinant GST expressed PI3K-C2α (See section 2.2.4.3) or wortmannin (5μM). The reaction was initiated by the addition of a mixture of (5μCi [γ-32P]ATP/assay, 5mM MgCl2, 10μM cold ATP), run for 20min at 25°C. The reaction was terminated by the addition of 4x
electrophoresis sample buffer and boiling. The reaction products were analysed by SDS-PAGE and either autoradiography or immunoblotting with specific antibodies.
3. Mechanisms involved in the regulation of class II PI3K activity

3.1 Summary

This chapter investigates the mechanisms involved in regulating the activity of PI3K-C2α. Phorbol esters are best recognised as activators of PKC and we demonstrate here that the phorbol ester PMA induces the activation of PI3K-C2α in CHO-IR, HEK 293 and L6 myoblasts, but not 3T3-L1 adipocytes, demonstrating a cell specific mechanism. This activation is similar in extent to that seen with insulin, while the inactive analog of PMA 4α-phorbol had no effect. By using a number of strategies such as overnight PMA treatment, the PKC inhibitor BIM-1, and the over-expression of a constitutively active PKCα construct, we demonstrate that the PMA-induced activation of PI3K-C2α is not directly regulated by PKC. Phorbol esters such as PMA are powerful activators of the ERK cascade; here we show, by using two chemical inhibitors of MEK (PD98059 and U0126) and the ste-MPKKPTPIQLNP cell permeable MEK derived peptide, that the PMA-induced, but not the insulin-induced activation of PI3K-C2α is ERK-dependent in CHO-IR cells. We found the same effects in L6 myoblasts after treating cells with PD98059, further confirming that this mechanism is not specific to CHO-IR cells. This is the first evidence for the involvement of the ERK cascade in the activation of a PI3K. In comparison, using the PI3K inhibitor wortmannin we demonstrate that the insulin-induced but not PMA-induced activation of PI3K-C2α is blocked by low doses of wortmannin in CHO-IR cells. This indicates that other classes of
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PI3K such as class IA or class III are involved in the insulin-induced activation of PI3K-C2α.

The activation of PI3K-C2α should lead to an increase in lipid content with the most likely lipid being PtdIns-3-P. Here we demonstrate in collaboration with the research groups of Dr Marco Falasca and Dr Frank Cooke that PMA induces an increase in PtdIns-3-P production in L6 cells that is ERK-dependent.

Studies of the effects of PMA and insulin on the association of tyrosine phosphorylated proteins with PI3K-C2α identified further differences in the way these two stimuli regulate PI3K-C2α. In particular PMA increased the tyrosine phosphorylated association of PI3K-C2α with a 120kDa protein, while insulin-induced PI3K-C2α to associate with a 160kDa protein (as we have previously reported (Brown, Domin et al., 1999)) and a 106kDa tyrosine phosphorylated protein. We provide evidence that the Focal Adhesion Kinase (FAK) and p130cas associate with PI3K-C2α, and are likely to contribute to the 120kDa tyrosine phosphorylated band. In conclusion we propose that PMA and insulin regulate PI3K-C2α by two separate pathways.

3.2 Introduction

A wide range of stimuli have been shown to activate the class II PI3Ks; these include activation by growth factors (Brown, Domin et al., 1999; Urso, Brown et al., 1999), integrins (Zhang, Banfic et al., 1998) and chemokines (Turner, Domin et al., 1998). Both PI3K-C2α and PI3K-C2β have been shown to be downstream signalling targets of activated EGF, PDGF receptors (Arcaro, Zvelebil et al., 2000) and insulin receptors (Brown, Domin et al., 1999; Urso, Brown et al., 1999; Brown and Shepherd
The functional role of PI3K-C2α is still controversial. Evidence has been presented claiming that the PI3K-C2α is involved in clathrin coated vesicle trafficking (Gaidarov, Smith et al., 2001; Prior and Clague 1999), activation of PKB (Arcaro, Khanzada et al., 2002) and nuclear mRNA processing (Didichenko and Thelen 2001). Recent reports also show a biological function for class II PI3K by expressing the protein during *Drosophila Melanogaster* development (MacDougall, Gagou et al., 2004). Results show that a class II polyproline motif in the catalytic subunit found in *Drosophila* and *C.elegans* (Vanhaesebroeck and Waterfield 1999c) (PI3K_68D) can bind the Drk adaptor protein *in vitro*, via the N-terminal SH3 domain of Drk, suggesting a role for Drk in the localisation of PI3K_68D, thus allowing Drk to modify signalling pathways downstream of cell surface receptors (MacDougall, Gagou et al., 2004).

We have set out to investigate how PI3K-C2α is regulated, and by which mechanisms. In these studies we have compared the effects of insulin with those of the phorbol ester PMA in the hope that this comparison would lead to the identification of pathways regulating PI3K-C2α. Phorbol esters are tumour promoting agents that were first shown to act through the stimulation of the PKC family of serine/threonine kinases (Buchner 1995). There are three different subclasses of PKC: conventional, atypical and novel PKCs (See introduction). In this present study, we investigated the role of PKC in the activation of PI3K-C2α by using a number of strategies including: (1) PKC inhibitors, (2) chronic treatment of cells with PMA that is known to downregulate PKC (Ishizuka, Cooper et al., 1991), and (3) transfection studies with constitutively active PKCα constructs in CHO-IR cells. It was thought for many years that the only receptors for phorbol esters were conventional or classical PKCs (cPKCs), and novel PKCs
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(nPKCs); however, not all the phorbol ester responses are through PKC. Additional phorbol ester receptors have been identified including: (1) mammalian α and β chimaerins, (2) Ras-GRP, and (3) Caenorhabditis elegans Unc-13 (Kazanietz 2002; Kazanietz 2000; Ron and Kazanietz 1999) (See Introduction), and these could be responsible for the effects of PMA on PI3K-C2α.

As PMA is a powerful activator of the ERK cascade we decided to test the involvement of ERK in the activation of PI3K-C2α. In this current study we investigated the role of ERK in the PMA-induced activation of PI3K-C2α by using three different strategies. The strategies included using: (1) a highly selective cell permeable chemical inhibitor of MEK (PD98059) that inhibits MEK1 activation and the MAPK cascade (Davies, Reddy et al., 2000; Dudley, Pang et al., 1995) (2) a cell permeable chemical inhibitor of MEK (U0126) that inhibits MEK1 and MEK2 activation (Davies, Reddy et al., 2000) and (3) a ste-MPKKKPTPIQLNP MEK derived peptide which blocks activation of ERK and which has been modified to allow cell permeability (Kelemen, Hsiao et al., 2002). We also decided to test whether other classes of PI3K were involved in the activation of PI3K-C2α. To do this we used the classic inhibitor of PI3K wortmannin at concentrations that are known to have little effect on PI3K-C2α but sufficient to block other isoforms of PI3K (Brown, Domin et al., 1999; Domin, Pages et al., 1997).

The activation of PI3K-C2α could lead to an increase in lipid content with PtdIns-3-P being the most likely lipid product. Both PMA and insulin have been shown to increase PtdIns-3-P levels in the cell (Maffucci, Brancaccio et al., 2003; Nave, Siddle et al., 1996; Stephens, Eguinoa et al., 1993). In collaboration with the research groups of Dr
Frank Cooke and Dr Marco Falasca, we conducted experiments to see whether an increase in PI3K-C2α activation was paralleled with an increase in PtdIns-3-P levels, and to also determine which signalling pathways were involved in this process.

In addition to being able to measure changes in activity in response to stimuli, we have previously observed that insulin induces association of PI3K-C2α with a 160kDa tyrosine phosphorylated protein (Brown, Domin et al., 1999). Such an association is very likely to have an effect on PI3K-C2α function so we have extended these studies to investigate the effects of PMA on the association with tyrosine phosphorylated proteins, and also to determine which signalling pathways might be involved in regulating any associations identified.

In the present study, we demonstrate that PMA and insulin induces activation of PI3K-C2α by two distinct pathways. Here we show that the PMA-induced activation of PI3K-C2α is ERK-dependent, but PKC-independent, and that the insulin-induced activation is dependent on other classes of PI3K such as class I or class III. We also demonstrate that PtdIns-3-P levels are increased in an ERK-dependent manner with increased activation of PI3K-C2α by PMA. Further we show that the 120kDa tyrosine phosphorylated band contains a member of the FAK and p130cas family of focal adhesion proteins. In conclusion we propose that PMA and insulin regulate PI3K-C2α by two separate pathways.
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3.3.1 Immune controls to show specificity of PI3K-C2α antisera generated

To test the specificity of the PI3K-C2α antibody generated as described in the Methods and Materials, non-stimulated CHO-IR cells were lysed and immunoprecipitation carried out with and without PI3K-C2α antisera as a control. The immunoprecipitates were then either subjected to SDS-PAGE (Figure 3.1 A), or a PI3K lipid kinase assay (Figure 3.1 B). Immunoprecipitates subjected to a lipid kinase assay were treated with 100nM wortmannin, or vehicle (DMSO) in vitro to further characterise that the product PtdIns-3-P produced was due to PI3K-C2α itself.

We found that PI3K-C2α was present in the total cell lysate and in the PI3K-C2α immunoprecipitate, but not in the immunoprecipitate without PI3K-C2α antisera (Figure 3.1 A). From this result we can conclude that the 180 kDa band that we see is due to PI3K-C2α itself, and not non-specific binding. Furthermore, we found that immunoprecipitates carried out with PI3K-C2α antisera generated the lipid product PtdIns-3-P that was not detected in immunoprecipitates without PI3K-C2α antisera (Figure 3.1 B). Further convincing evidence came from the result that the lipid produced was not blocked by the PI3K inhibitor wortmannin at 100nM. Such data provided evidence that PI3K-C2α was responsible for making this lipid, as PI3K-C2α is the only known class of PI3K to be resistant to this concentration of wortmannin (Figure 3.1 B).
3.3.2 PMA activates PI3K-C2α in vitro and in vivo in a cell-specific manner

In order to investigate the role of ERK and PKC in the pathways regulating PI3K-C2α activity we chose to look at insulin and PMA as both are potent stimulators of the ERK and PKC cascade in many cell types. PMA was found to induce the activation of PI3K-C2α in CHO-IR, HEK 293 cells and L6 myoblasts, but not 3T3-L1 adipocytes (Figure 3.2). The extent of the PMA activation was similar to that seen with insulin (Figure 3.2). Insulin caused activation of PI3K-C2α in CHO-IR, HEK 293 cells, L6 myoblasts and 3T3-L1 adipocytes with maximum stimulation approximately 3.5-fold in 3T3-L1 adipocytes cells. The inactive analog of PMA, 4-α-phorbol (Figure 3.3 Structure comparison of PMA and 4-α-phorbol) had no effect on PI3K-C2α activation or phosphorylation of ERK in vivo indicating that the effects of PMA were specific (Figure 3.4).

In CHO-IR cells a time-course of PMA treatment in vivo was carried out to find the optimal time for stimulation. Results showed that the activation by PMA persists for at least 30mins with maximal stimulation at 10minutes (Figure 3.5). We also compared the affects of PMA on PI3K-C2β activation in CHO-IR cells, and found that PMA did not stimulate PI3K-C2β activation, suggesting that PMA regulation of class II PI3K is isoform specific (Figure 3.6). Surprisingly, after 15mins stimulation with PMA we observed a reproducible decrease in the activity of PI3K-C2β that was seen in all experiments carried out. After 20mins stimulation the activity levels increased back to that seen after 10mins stimulation. As well as testing the effects of PMA on PI3K-C2α activity in vivo we tested the affects in vitro in CHO-IR cells. We found that PI3K-C2α was activated by PMA in vitro at concentrations lower than that seen for the
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stimulation of PI3K-C2α \textit{in vivo} (Figure 3.7). These results at first would suggest that the effects we see of PMA \textit{in vivo} are due to either PMA directly binding to PI3K-C2α and modulating its activity, or that PMA is causing a change in the lipid environment. If this was the case then we would expect to see increases in the activation of PI3K-C2α by PMA in all cell types for example 3T3-L1 adipocytes (Figure 3.2); however, we do not, hence the effects must be specific. Furthermore, the PMA used to stimulate the cells would have been washed away after carrying out multiple washes of the immunoprecipitates, thus the effects observed are not due to perturbation of the lipid environment. We found pre-treatment with PMA did not increase the maximal effect of insulin, and neither did the insulin pre-treatment affect the activation imposed by PMA in CHO-IR cells, suggesting that both stimuli might be targeting the same mechanism of activation (Figure 3.8).

3.3.3 PMA regulation of PI3K-C2α activity is independent of PKC

As we have already found that PMA directly regulates PI3K-C2α we decided to test whether the PMA-induced activation of PI3K-C2α required PKC for its activation. To do this we used three different strategies in CHO-IR cells: (1) the PKC inhibitor BIM-1 to look at the effects this had on phorbol ester activation of PI3K-C2α; (2) the chronic overnight treatment of cells with PMA, which downregulates cellular PKC levels (Ishizuka, Cooper et al., 1991); and (3) the overexpression of a constitutively active PKCα construct. Phorbol ester activation is classically taken to indicate the involvement of PKC, but this does not seem to be the case for activation of PI3K-C2α as treatment of cells with the PKC inhibitor BIM-1 had no effect on the activation induced by PMA
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(Figure 3.9 A). We show that overnight treatment of cells with PMA down-regulated PKC α/β protein levels (Figure 3.9 B), but did not reduce the activation of PI3K-C2α (Figure 3.9 C) suggesting that PKC is not directly involved in the activation of PI3K-C2α. We further show that PI3K-C2α protein levels (see chapter 4) do not change on PMA treatment, further confirming that the effects that we see represent changes in activity and not changes in PI3K-C2α protein levels. To further illustrate the involvement of PKC in the regulation of class II PI3K we tested the effects of transfecting a constitutively active PKCα construct into CHO-IR cells. This resulted in clear increases in PKC expression (Figure 3.10). We found that transfection of the constitutively active PKCα construct had no affect on PI3K-C2α activity before or after PMA stimulation, suggesting that PI3K-C2α activity is at least not regulated by PKCα (Figure 3.10 A). Surprisingly in all experiments carried out we observed a reduction in the total level of PKC in cells transfected with PKC, and then stimulated with PMA. At this stage we cannot draw any conclusions from the data and further experiments would be required. Transfection efficiency was proved by detection of over-expressed PKC from lysates where PKC was transfected, compared to mock-transfected, and non-transfected cells (Figure 3.10 B).

3.3.4 PMA-induced activation of PI3K-C2α is regulated by ERK whereas insulin-induced activation is regulated by other classes of PI3K.

While insulin and PMA activate PI3K-C2α, the mechanism by which this activation is achieved remains unclear; therefore, we decided to look at the effects of ERK as a candidate pathway involved. We found that PMA stimulation in CHO-IR cells lead to
strong and sustained activation of ERK1 and ERK2 kinases while insulin caused a smaller and transient activation (Figure 3.11 A). Two highly specific cell permeable inhibitors of MEK, PD98059 and U0126 blocked this activation (Figure 3.11 A+B). Using PD98059 we found that the insulin activation of PI3K-C2α was not blocked by PD98059 (Figure 3.12 A). Using PD98059, U0126 and the ste-MP KK PTPIQLNP cell permeable MEK derived peptide in CHO-IR cells, we found that the activation of PI3K-C2α by PMA (Figure 3.12 A, B and C) was blocked. The same effects were observed in L6 myoblasts after using PD98059, further suggesting that this effect was caused by inhibition of ERK (Figure 3.12 A). These results suggest that insulin and PMA regulate PI3K-C2α by two distinct mechanisms.

To test whether ERK might directly activate PI3K-C2α we tested the effect of recombinant active ERK on the activation of PI3K-C2α in vitro. We added recombinant active ERK to PI3K-C2α immunoprecipitates in vitro, however we were unable to show any affect on PI3K-C2α kinase activity from CHO-IR cells or 3T3-L1 adipocytes (Figure 3.13). However, in retrospect the design of these experiments was flawed as they were performed on PI3K-C2α immunoprecipitates, and the antibody used contains the putative ERK site, thus any effect on the activation would be difficult to detect (see chapter 4 for more information). As the INK signalling cascade is very similar to the ERK cascade and previous reports have shown that INK phosphorylates PI3K-C2α (Didichenko, Fragoso et al., 2003) we decided to look at whether this pathway was also involved in the regulation of PI3K-C2α. however, insulin- and PMA-induced activation of PI3K-C2α was very weak in this experiment therefore no clear conclusions could be drawn (See Figure 3.14).
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To investigate whether other forms of PI3K might be involved in the regulation of PI3K-C2α, we used the PI3K inhibitor wortmannin. At a concentration of 100nM, wortmannin has minimal effects on the activity of PI3K-C2α, but would be sufficient to inhibit the activity of all other PI3K isoforms (Brown, Domin et al., 1999; Domin, Pages et al., 1997). We investigated the effects on both the insulin- and PMA-induced activation of PI3K-C2α in CHO-IR cells. 100nM wortmannin completely blocked the insulin-induced activation of PI3K-C2α but had no effect on the PMA-induced activation (Figure 3.15). Mammalian target of rapamycin (mTOR) is downstream of PI3K so we decided to test the effects of the specific mTOR inhibitor rapamycin on both the insulin- and PMA-induced activation of PI3K-C2α in CHO-IR cells. We found that rapamycin had no affect on either, demonstrating that mTOR was not involved (Figure 3.16).

3.3.5 PMA induces PtdIns-3-P formation in L6 cells and is blocked by PD98059.

Work was carried out in collaboration with the research groups of Dr Marco Falasca and Dr Frank Cooke to investigate changes in cellular lipid levels. The activation of PI3K-C2α should lead to an increase in its lipid product, most likely PtdIns-3-P. PtdIns-3-P has been shown to be increased by insulin and PMA (Maffucci, Brancaccio et al., 2003; Nave, Siddle et al., 1996; Stephens, Eguinoa et al., 1993). We decided to test the effects of the ERK cascade on the PMA-induced PtdIns-3-P levels in L6 cells. In collaboration we used HPLC analysis, confocal microscopy and quantitative analysis to test these effects. For confocal microscopy and analysis, L6 cells were transfected with a cDNA encoding the double FYVE domain from the hepatocyte-growth-factor-regulated tyrosine
kinase substrate (Hrs) fused to the green fluorescent protein (GFP-2XFYVE\textsuperscript{int}) which is a specific probe for PtdIns-3-P (Gillooly, Morrow et al., 2000). After pre-incubating cells with the MEK inhibitor PD98059 cells were stimulated with PMA and the localisation of the FYVE finger domain was detected using confocal microscopy. We found a direct increase in PtdIns-3-P production using HPLC (Figure 3.17 A) and an indirect increase in PtdIns-3-P production using confocal microscopy (Figure 3.17 B, C and D) in L6 myoblasts after PMA stimulation. Under these conditions PMA had no effect on PtdIns-3,4-P\textsubscript{2} or PtdIns-3,4,5-P\textsubscript{3} levels (data not shown). Furthermore, we found that the PMA-induced increase in PtdIns-3-P levels was only partially blocked by the ERK inhibitor PD98059 (Figure 3.17 A, C and D) compared to the activation of PI3K-C2\textalpha{} (Figure 3.12 A). This result suggests that the PMA-induced increase in PtdIns-3-P levels is at least partially ERK-dependent.

3.3.6 Association of PI3K-C2\textalpha{} with tyrosine phosphorylated proteins after insulin and PMA stimulation.

The studies described above indicate that insulin and PMA both use different pathways to regulate the activity of PI3K-C2\textalpha{}. This was further supported by the finding that insulin and PMA stimulated association of PI3K-C2\textalpha{} with different tyrosine phosphorylated proteins in CHO-IR cells. Insulin-stimulated association of PI3K-C2\textalpha{} with a 160kDa phosphotyrosine protein as we have previously described (Brown, Domin et al., 1999), and a 106kDa phosphotyrosine protein (Figure 3.18). Conversely PMA, but not insulin, increased association with an unknown 120kDa tyrosine-phosphorylated protein (Figure 3.18). To determine if ERK was required for the association of PI3K-C2\textalpha{} with these
tyrosine phosphorylated proteins in CHO-IR cells, we tested the effects of the MEK inhibitor PD98059. However, we were unable to show any significant changes in association upon PD98059 treatment (Figure 3.18).

In comparison we decided to test the effects of the PI3K inhibitor wortmannin on the association of PI3K-C2α with tyrosine phosphorylated proteins in CHO-IR cells. Although we found that wortmannin blocks the insulin-induced activation of PI3K-C2α, we were unable to show any change in the insulin-induced association of PI3K-C2α with the 160kDa or 106kDa tyrosine phosphorylated proteins (Figure 3.19). Furthermore, we found no change in the PMA-induced association of PI3K-C2α with the 120kDa tyrosine phosphorylated protein either (Figure 3.19). Surprisingly in these experiments carried out we were unable to detect any increase in association of PI3K-C2α with the 120kDa tyrosine phosphorylated protein as seen in Figure 3.18. At this point it is hard to conclude if any changes occur to the 120kDa protein therefore further investigations would be required.

The molecular weight of the 106kDa tyrosine phosphorylated protein and the fact that it associates with PI3K-C2α after insulin stimulation, led us to believe that the candidate protein could be the β subunit of the insulin receptor. However, we were unable to confirm that this protein is the β subunit of the insulin receptor as the available antibodies against this protein are poor. Possible candidates for the 120kDa tyrosine phosphorylated protein include cbl, Gab1 and the family of focal adhesion proteins FAK, Pyk2 and p130cas as they have similar molecular weights to the protein in question, and are tyrosine phosphorylated by diverse stimuli. To determine if any of these candidates were authentic binding partners of PI3K-C2α, we immunoprecipitated PI3K-C2α from cells.
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stimulated with insulin or PMA and western blotted with cbl, Gab1, FAK, Pyk2 and p130
Cas specific antisera. We were unable to show by western blotting any association of
PI3K-C2α with cbl-b (Figure 3.20 A); cbl was present in the cell lysates (Figure 3.20 A)
which confirmed that the cbl-b antibody was working. In the case of Gab1 or Pyk2 we
cannot reach a definite conclusion as the antibodies we used were poor at detecting either
of these proteins in cell lysates from CHO-IR. Surprisingly we found that the focal
adhesion proteins FAK and p130Cas were constitutively associated with PI3K-C2α after
both insulin and PMA activation (Figure 3.20 B). The identities of the other
phosphotyrosine proteins to date remain unknown, further investigations would be
required to identify these proteins.

3.4 Discussion

In an effort to identify signalling mechanisms involved in regulating PI3K-
C2α, we focussed on comparing its regulation by both phorbol esters and insulin in a
wide range of cell types. Many endpoint responses stimulated by phorbol esters overlap
to some extent with responses activated by growth factors, for example gene expression
(Buchner 1995), membrane trafficking (Gibbs, Calderhead et al., 1991; Tanaka and
Nishizuka 1994) and membrane ruffling (Kotani, Yonezawa et al., 1994). Here we have
added a number of agents that induce the activation of PI3K-C2α to the list of agents that
are already known to regulate class II PI3K.

Phorbol esters are traditionally associated with the activation of classical PKC
isoforms; however we find inhibition of PKCs using several different strategies does not
block activation of PI3K-C2α. Further the overexpression of a constitutively active
PKCα construct does not change the PMA-induced activation of PI3K-C2α. This
indicates that either; one of the other recently identified phorbol ester targets is the
mediator of the PMA signal (Brose and Rosenmund 2002; Kazanietz 2002; Kazanietz
2000; Ron and Kazanietz 1999), or that it does not require PKC kinase activity. PMA is
also a powerful indirect activator of the ERK pathway in many cell types, and using
several different strategies we show that activation of ERK is required for phorbol ester-
induced activation of PI3K-C2α. These data provide the first evidence that the ERK
pathway is directly involved in the activation of a member of the PI3K family.
Interestingly, using Scansite (scansite.mit.edu), which finds potential phosphorylation
sites as well as binding sites within a specific sequence, we found two potential ERK1
kinase phosphorylation sites as well as an ERK1 binding site in the protein sequence of
PI3K-C2α; this further suggests the involvement of ERK in the regulation of PI3K-
C2α (for more information see chapter 4). In comparison the insulin-induced activation
of PI3K-C2α is not blocked by the inhibition of ERK activation, but is blocked by
100nM wortmannin. As 100 nM wortmannin has little effect on the enzymatic activity of
PI3K-C2α itself (Brown, Domin et al., 1999; Domin, Pages et al., 1997), these data
indicate that insulin activates PI3K-C2α by a pathway that probably requires the
activation of a class I or class III PI3K. Cell type differences in expression of the
components of these two signalling pathways is likely to explain the cell specific patterns
of activation seen for PI3K-C2α. This is exemplified in 3T3-L1 adipocytes where insulin
activates PI3K-C2α, but PMA does not, despite the fact that PMA is reported to activate
ERK in adipocytes (MacKenzie, Fleming et al., 1997; Haystead, Gregory et al., 1994),
and suggests that 3T3-L1 adipocytes lack a signalling component downstream of ERK that is critical for ERK-dependent activation of PI3K-C2α.

One pathway downstream of class I PI3K is mTOR and this pathway can be blocked by using the specific mTOR inhibitor rapamycin. We decided to test the effects of rapamycin on the activation of PI3K-C2α because rapamycin has been shown to inhibit PI3K-induced cell migration (Qian, Corum et al., 2004; G-Cambronero 2003), and it is known that PI3K-C2α is involved in smooth muscle cell migration (Paulhe, Perret et al., 2002). These reports show that PI3K-C2α is differentially regulated by α3β1 and α5β1 integrin engagement (Paulhe, Perret et al., 2002). However, rapamycin had no effect on either insulin- or PMA-induced activation of PI3K-C2α.

The activation of PI3K-C2α should lead to the increase in lipid content in the cell most likely PtdIns-3-P. It is well established that PtdIns-3-P is localized on endosomes (Gillooly, Morrow et al., 2000). The most important role of PtdIns-3-P appears to be in regulating vesicle trafficking. A number of effectors of PtdIns-3-P have been identified, most containing FYVE finger domains (Driscoll and Vuidepot 1999) or PX domains (Yu and Lemmon 2001). However, only recently have transient changes in PtdIns-3-P been demonstrated, in response to external stimuli, and one important cellular process regulated by changes in PtdIns-3-P, is the translocation of GLUT4 glucose transporters (Maffucci, Brancaccio et al., 2003).

PI3K-C2α is known to be resistant to wortmannin (Domin, Pages et al., 1997; Virbasius, Guilherme et al., 1996), and recent reports show that the insulin dependent pool of PtdIns-3-P in L6 cells is also resistant to wortmannin (Maffucci, Brancaccio et al., 2003), which further indicates that PtdIns-3-P is the lipid product of PI3K-C2α. Both
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PMA and insulin have been shown to increase cellular PtdIns-3-P levels (Maffucci, Brancaccio et al., 2003; Nave, Siddle et al., 1996; Stephens, Eguinoa et al., 1993). The phorbol ester stimulation of PtdIns-3-P production cannot be explained by the activation of class-I PI3K (Herbert, Kilhmas et al., 2000; Nave, Siddle et al., 1996; Stephens, Jackson et al., 1993), suggesting that class II PI3K could be involved. Given our finding that PI3K-C2α is activated by PMA it seemed logical to investigate the effects of PMA on PtdIns-3-P in systems used in the current study. In studies performed in collaboration with the groups of Dr Marco Falasca and Dr Frank Cooke, we found that PMA-stimulated both an increase in PtdIns-3-P lipid levels, and the translocation of FYVE finger domains to the plasma membrane. To date there are no reports of growth factor-stimulated production of PtdIns-3-P at the plasma membrane, except for a recent publication from our collaborators where they showed that insulin induced the translocation of PtdIns-3-P to the plasma membrane (Maffucci, Brancaccio et al., 2003). What was more interesting and exciting was our finding that PMA as well as insulin induces the translocation of PtdIns-3-P to the plasma membrane. Furthermore, this PMA-induced translocation of PtdIns-3-P was found to be ERK-dependent, which suggested a role for ERK in this regulation. In conclusion we have shown in this chapter that PMA stimulation of 3T3-L1 adipocytes does not induce the activation of PI3K-C2α, or the increase of PtdIns-3-P production (Nave, Siddle et al., 1996). These studies together are consistent with the hypothesis that effects of phorbol esters on PtdIns-3-P production are due to the actions of PI3K-C2α.

In summary our findings suggest that either ERK phosphorylates PI3K-C2α, or activates some downstream signalling cascade that causes activation of PI3K-C2α. In
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Chapter 4 the link between ERK and PI3K-C2α phosphorylation is investigated and the link between ERK and PI3K-C2α activation is discussed in the final discussion chapter.

One aspect of regulation that will be discussed in this chapter is the issue of the proteins that bind to PI3K-C2α. There are differences in the regulation of PI3K-C2α and PI3K-C2β (Didichenko and Thelen 2001; Brown, Domin et al., 1999; Crljen, Volinia et al., 2002; Zhang, Banfic et al., 1998), and it is possible that the basis of the difference in the signalling pathways regulating PI3K-C2α and PI3K-C2β could lie in the formation of different signalling complexes. For example EGF stimulates the association of both PI3K-C2α and PI3K-C2β with the EGF-Receptor (Arcaro, Zvelebil et al., 2000), PI3K-C2β associates with grb2 (Wheeler and Domin 2001), PI3K-C2α associates with clathrin heavy chain (Gaidarov, Smith et al., 2001; Prior and Clague 1999), and insulin stimulates association of PI3K-C2α with an unknown 160kDa protein (Brown, Domin et al., 1999).

In the current study we have chosen to focus on the use of anti-phosphotyrosine antibodies to identify tyrosine phosphorylated proteins present in PI3K-C2α immunoprecipitates. In the current studies we have identified clear differences in the pattern of tyrosine phosphorylation in these immunoprecipitates after cells have been stimulated with either PMA, or insulin. Such changes indicate that either the tyrosine phosphorylated proteins are being recruited to PI3K-C2α, or that the proteins constitutively associated with PI3K-C2α are becoming tyrosine phosphorylated. In our studies we find a tyrosine phosphorylated band of 120kDa, that increases in PI3K-C2α immunoprecipitates following PMA stimulation, while increases in the tyrosine phosphorylated proteins of 160kDa and 106kDa are observed following insulin stimulation. Here in this thesis we have tried to identify these protein by testing candidate
proteins. We have also proposed the identity of the proteins for the reasons listed below. Candidates for the tyrosine phosphorylated proteins are as follows;

(i) Possible candidates for the 160kDa tyrosine phosphorylated protein that associates with PI3K-C2\(\alpha\) after insulin stimulation

Candidate proteins for the 160kDa tyrosine phosphorylated protein include the IRS family of proteins such as IRS-1. However, work carried out by Dr Richard Brown (a previous member of the Shepherd laboratory) found that there was no IRS-1 or IRS-2 protein present in PI3K-C2\(\alpha\) immunoprecipitates from stimulated CHO-IR cells, or from IRS-1 immunoprecipitates from cells transfected with Myc-tagged IRS-1. Other candidates for the 160kDa tyrosine phosphorylated protein include the SHIP family of proteins most likely SHIP2 which is ubiquitously expressed (Backers, Blero et al., 2003; Pesesse, Deleu et al., 1997). These proteins are tyrosine phosphorylated by distinct stimuli such as growth factors and cytokines but have slightly lower molecular weights (145kDa) than the protein we observe to be associated with PI3K-C2\(\alpha\) after insulin stimulation. The apparent molecular weight of this protein on SDS-PAGE might well be 160kDa, for example, due to phosphorylation, however, further investigations would be required.

(ii) Possible candidates for the 106kDa tyrosine phosphorylated protein that associates with PI3K-C2\(\alpha\) after insulin stimulation

Candidates for the 106kDa tyrosine phosphorylated protein include the \(\beta\) subunit of the insulin receptor. The \(\beta\) subunit of the insulin receptor is 106kDa, and also associates with
PI3K-C2α after insulin stimulation. However, we were unable to show that this protein was the insulin receptor.

(iii) Possible candidates for the 120kDa tyrosine phosphorylated protein that associates with PI3K-C2α after PMA stimulation

With the 120kDa tyrosine phosphorylated protein there were a number of candidates that we thought could be the protein. At first we thought it was one of the members of the cbl protein family, which have been implicated in RTK signalling, and have been shown to undergo both tyrosine phosphorylation, and membrane translocation in response to insulin in 3T3-L1 adipocytes (Liu, DeYoung et al., 2003). The cbl protein has the same molecular weight as the protein we find to associate with PI3K-C2α after PMA stimulation. Furthermore, cbl is known to bind the p85 subunit of PI3K and Grb2 (Gesbert, Garbay et al., 1998), which is an adaptor recently found to be associated with PI3K-C2β (Gaidarov, Smith et al., 2001). However, western blot analysis would indicate that the 120kDa protein was not cbl. Other candidates for the 120 kDa protein include the Gab family of proteins, for example Gab1. Reasons for the Gab1 protein being a possible candidate include the fact that the protein is tyrosine phosphorylated in response to stimuli such as growth factors and cytokines, it has the same molecular weight as the protein in question and it also binds Grb2 (Gu and Neel 2003; Liu and Rohrschneider 2002). Furthermore, Grb2 was recently found to be associated with PI3K-C2β (Gaidarov, Smith et al., 2001). However, western blot analysis was inconclusive due to poor immunoreactivity of the antibodies available.
Other possible candidates for the 120kDa protein are members of the focal adhesion family of proteins such as: Focal Adhesion Kinase (FAK) (Avraham, Park et al., 2000; Parsons 2003); RAFTK/Pyk2; and p130cas (Avraham, Park et al., 2000). These proteins have molecular weights ranging from 125-130kDa, and play a key role at sites of cellular adhesion. They have been shown to be associated with PI3K in NIH 3T3 mouse fibroblasts (Chen and Guan 1994). Reasons for the focal adhesion proteins FAK, RAFTK/Pyk2 and p130cas being possible candidates include: they have similar molecular weights to the unknown 120kDa tyrosine phosphorylated protein; they are regulated by diverse stimuli including growth factors such as insulin; and they are activated and phosphorylated by integrins (Zachary and Rozengurt 1992) as is PI3K-C2α (Paulhe, Perret et al., 2002). PMA has also previously been shown to increase Pyk2 expression in Jurkat T cells and normal T cells hence involving the PKC-ERK cascade (Tsuchida, Manthei et al., 1999). The FAK protein has further been shown to be associated with Grb2, which results in the activation of MAPK (Igishi, Fukuhara et al., 1999; Schlaepfer, Hanks et al., 1994.). We provide evidence that FAK and p130cas associate with PI3K-C2α and are likely to contribute to the 120kDa tyrosine phosphorylated band observed. However, it is unlikely that PI3K-C2α directly binds to p130 cas due to the intensity of the band that we observe which is considerably higher in cell lysates compared to the PI3K-C2α immunoprecipitates. It is more likely that FAK and p130 cas form a complex and that the association with PI3K-C2α is indirect. To further confirm our data recent reports show that high concentrations of wortmannin (1000nM) are needed to inhibit tyrosine phosphorylation of p130cas and FAK in rat pancreatic acini (Rosado, Salido et al., 2000). This concentration of wortmannin is also
required to block PI3K-C2α. Furthermore, it has been shown that only wortmannin-resistant PI3K activity associates with FAK (Reiske, Kao et al., 1999), implicating PI3K-C2α as this activity. The role of these tyrosine phosphorylated proteins in regulating PI3K-C2α activity is not known, but the differences in association with PI3K-C2α are likely to be indicative of different functional output. Further work would be required to identify the other tyrosine phosphorylated proteins associated with PI3K-C2α, and the mechanisms by which these proteins regulate PI3K-C2α activity.

In conclusion our studies provide evidence that indicates that there are at least two distinct signalling pathways involved in the regulation of the intrinsic activity of PI3K-C2α. In this study we also provide the first direct evidence that ERK is required for pathways regulating PI3K-C2α activity in the cell. These observations should provoke further work in this area, and should contribute to our understanding of the PI3K family.
Figure 3.1 Specificity of PI3K-C2α antisera generated.

CHO-IR cells were lysed, and lysates (1mg) were subjected to immunoprecipitation with and without purified PI3K-C2α antisera (SK1493).

PI3K-C2α immunoprecipitates, lysates without beads denoted beads only, and 50µg of total lysate were subjected to SDS-PAGE, and Western blotted with purified SK1493 serum (1:500) (A). Similar results were obtained in at least 3 separate experiments. PI3K-C2α immunoprecipitates, and immunoprecipitates without PI3K-C2α (denoted beads only) were also treated with 100nM wortmannin in vitro for 15mins and then PI3K assays were performed on these immunoprecipitates. B is a TLC of an autoradiograph. Again similar results were obtained in at least 3 separate experiments.
Figure 3.2 Effects of insulin and PMA on PI3K-C2α activation in CHO-IR, HEK 293, 3T3-L1 adipocytes and L6 myoblasts.

Cells were serum-starved overnight and then stimulated with 100nM insulin for 5mins, or 1μM PMA for 10mins. Cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2α specific antisera. PI3K assays were performed on these immunoprecipitates. Results are the means of five experiments carried out in duplicate for CHO-IR cells, two experiments carried out in triplicate for HEK 293 cells, four experiments carried out in duplicate for 3T3-L1 adipocytes, and three experiments carried out in duplicate for L6 myoblasts. Basal values were normalised within each cell type. Results are expressed as % of basal (± S.E.M). P-values were calculated using the student t-test where p≤0.01 (**) or where p≤0.05 (*) compared to basal unless otherwise stated. Where there are no p-values data did not reach statistical significance.
Fig 3.3 Structure comparison of PMA and 4-\(\alpha\)-phorbol.

PMA is a tumour promoting agent that activates PKC \textit{in vivo} and \textit{in vitro} even at nanomolar concentrations. 4-\(\alpha\)-phorbol is the structurally related ester that acts to activate PKC. 4-\(\alpha\)-phorbol is used to control for non-specific actions of PMA.

The figure shows the structural differences in the two esters. Highlighted area is the area which the two compounds differ in structure.
Figure 3.4 The inactive analogue of PMA, 4α-phorbol does not increase ERK phosphorylation or activate PI3K-C2α in CHO-IR cells.

CHO-IR cells were serum-starved overnight and then incubated with 50μM MEK1 inhibitor PD98059 for 30mins, followed by stimulation with either 1μM PMA or 1μM 4α-phorbol for 10 minutes. Cells were lysed, and 50μg cell lysate were subjected to SDS-PAGE and Western blotted with phospho and total ERK specific antisera (A). Similar results were obtained in at least 3 separate experiments. Lysates were subjected to immunoprecipitation using PI3K-C2α specific antisera and PI3K assays were performed on these immunoprecipitates. B is a TLC of an autoradiograph. Results are the means of three experiments carried out in duplicate and are expressed as % of basal. Where there are no p-values data did not reach statistical analysis.
Figure 3.5 Time-course effects of PMA on PI3K-C2α activity in CHO-IR cells.

CHO-IR cells were serum-starved overnight and then stimulated with 1μM PMA for indicated time-points. Cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2α specific antisera. PI3K assays were performed on these immunoprecipitates. Results are the means of two experiments carried out in triplicate for CHO-IR cells. Results are expressed as % of basal (+/ - S.E.M). Basal values were normalised and p-values were calculated using the student t-test. Where there are no p-values data did not reach statistical significance.
Figure 3.6 Time-course effects of PMA on PI3K-C2β activity in CHO-IR cells.

CHO-IR cells were serum-starved overnight and then stimulated with 1μM PMA for indicated time-points. Cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2β specific antisera. PI3K assays were performed on these immunoprecipitates. Results are the means of two experiments carried out in triplicate for CHO-IR cells. Results are expressed as % of basal (+/ - S.E.M). Basal values were normalised and p-values were calculated using the student t-test. Where there are no p-values data did not reach statistical significance.
Figure 3.7 *In vitro* effects of PMA on PI3K-C2α activity in CHO-IR cells.

CHO-IR cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2α specific antisera. Immunoprecipitates were incubated with various concentrations of PMA *in vitro*. PI3K assays were performed on these immunoprecipitates. Results are the means of five experiments carried out in duplicate. Results are expressed as % of basal (+/- S.E.M). Basal values were normalised and p-values were calculated using the student t-test where p≤0.01 (**) or where p≤0.05 (*) compared to basal unless otherwise stated. Where there are no p-values data did not reach statistical significance.
Figure 3.8 Effects of insulin and PMA on PI3K-C2α activity in CHO-IR cells.

CHO-IR cells were serum-starved overnight and then stimulated sequentially with 100nM insulin for 5mins, or and 1μM PMA for 10mins, or vice-versa as indicated. Cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2α specific antisera. PI3K assays were performed on these immunoprecipitates. Results are the means of four experiments carried out in duplicate. Results are expressed as % of basal (± S.E.M). Basal values were normalised and p-values were calculated using the student t-test where $p \leq 0.01$ (** or where $p \leq 0.05$ (*) compared to basal unless otherwise stated. Where there are no p-values data did not reach statistical significance.
Chapter 3

A

Cell lysates

| Insulin | + | - | - | - | - | + | - |
| PMA | - | - | + | - | - | - | + |
| PMA O/N | - | - | - | + | + | + | + |

p-PKCα/βII

B

PI3K activity (% of basal)

Basal | Insulin | PMA | PMA O/N | PMA O/N + Ins | PMA O/N + PMA

C

Treatment of cells

PI3K activity (% of basal)

Basal | BIM-1 | PMA | PMA + BIM-1
Figure 3.9 PMA stimulation of PI3K-C2α is not directly regulated by PKC in CHO-IR cells.

CHO-IR cells were serum-starved overnight and then stimulated with 100nM insulin for 5mins, 1μM PMA for 10mins, or incubated with 1μM PMA overnight then stimulated with 100nM insulin for 5mins, or 1μM PMA for 10mins (A +B). Cells were preincubated with 5μM BIM-1 for 15mins followed by stimulation with 1μM PMA for 10mins (C). Cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2α specific antisera. PI3K assays were performed on these immunoprecipitates (B+C). 50μg of protein lysate was subjected to SDS-PAGE and blotted with phospho PKCα/βII (A). Results are the means of four experiments carried out in duplicate (B+C) and three experiments carried out in triplicate (A). Results are expressed as % of basal (+/- S.E.M). Basal values were normalised and p-values were calculated using the student t-test where p≤0.01 (**) or where p≤0.05 (*) compared to basal unless otherwise stated. Where there are no p-values data did not reach statistical significance.
PI3K activity (% of basal)

A

Treatment of cells

Mock TF Non- TF Non- TF + PMA PKC PKC + PMA

B

NT NT +P MT PKC PKC+P

p- PKC α/β II

PKC
Figure 3.10 Effects of transfecting a constitutively active PKC construct on PI3K-C2α activation in CHO-IR cells.

CHO-IR cells were sub-cultured into 6 well plates and transfected with using LipofectAMINE (5μl), and plasmid DNA (constitutively active PKC) (2μg per well in 6well dish). Protein expression from the transfected plasmids was found to be optimal 24h post-transfection. Cells were serum starved 3hours and then stimulated with 1μM PMA for 10mins. Cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2α specific antisera. PI3K assays were performed on these immunoprecipitates (A). Results are the means of three experiments carried out in triplicate. Results are expressed as % of basal (+/ - S.E.M). Cell lysates (20μg) were subjected to SDS-PAGE and Western blotted with phospho and total PKC specific antisera (B). NT= non transfected and MT= mock transfected. Similar results were obtained in at least three experiments. Basal values were normalised and p-values were calculated using the student t-test. Where there are no p-values data did not reach statistical significance.
Chapter 3

Results

A

PD98059

Control | Insulin | PMA

-       | +       | -       | +       | -       | +       |

p-ERK

ERK

p-MEK

MEK

B

Basal | PMA

-       | +       | -       | +       |

U0126

p-ERK

ERK

p-MEK

MEK
Figure 3.11 Effects of MEK inhibitors on insulin- and PMA-induced ERK and MEK phosphorylation in CHO-IR cells.

CHO-IR cells were serum-starved overnight, and then incubated with inhibitors for 30 mins (50 μM PD98059 (A) or 10μM U0126 (B)), followed by stimulation with either 1μM PMA for 10 mins, or 100nM insulin for 5 mins as indicated. Cells were lysed and 50μg cell lysate was subjected to SDS-PAGE and western blotted with either phospho or total ERK and MEK specific antisera. Similar results were obtained in at least three experiments.
Chapter 3

Results

A

PI3K activity (% of basal)

CHO-IR cells
L6 myoblasts

PD98059

B

PI3K activity (% of basal)

UO126

C

PI3K activity (% of basal)

Ste-MEK1

U0126

PMA

Treatment of cells

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Chapter 3

Results

Figure 3.12 Effects of MEK inhibitors on insulin- and PMA-induced activation of PI3K-C2α in CHO-IR and L6 myoblast cells.

CHO-IR cells were serum-starved overnight and then incubated with inhibitors for 30 mins (50 μM PD98059 CHO-IR and L6 myoblasts (A), 10μM U0126 CHO-IR only (B) and 50μM cell permeable MEK derived peptide CHO-IR only (C)), followed by stimulation with either 1μM PMA for 10mins or 100nM insulin for 5mins as indicated. Cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2α specific antisera. PI3K assays were performed on these immunoprecipitates. Results are the means of at least three experiments carried out in duplicate and are expressed as % of basal (+/- S.E.M). Basal values were normalised and p-values were calculated using the student t-test where p≤0.01 (**) or where p≤0.05 (*) compared to basal unless otherwise stated. Where there are no p-values data did not reach statistical significance.
Figure 3.13 *In vitro* effect of recombinant active ERK on PI3K-C2α activity in CHO-IR and 3T3-L1 adipocytes.

CHO-IR and 3T3-L1 adipocyte cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2α specific antisera. Recombinant active ERK was added to immunoprecipitaes as indicated for 15mins. PI3K assays were performed on these immunoprecipitates. Results are the means of two experiments carried out in triplicate. Results are expressed as % of basal (+/- S.E.M). Basal values were normalised and p-values were calculated using the student t-test. Where there are no p-values data did not reach statistical significance.
Figure 3.14 Effects of the JNK inhibitor SP600125 on insulin- and PMA-induced activation of PI3K-C2α in CHO-IR cells.

CHO-IR cells were serum-starved overnight and then incubated with 25 μM SP600125 for 30 mins followed by stimulation with either 1 μM PMA, for 10 mins, or 100 nM insulin for 5 mins as indicated. Cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2α specific antisera. PI3K assays were performed on these immunoprecipitates. Results are the means of three experiments carried out in duplicate and are expressed as % of basal (+/- S.E.M). Basal values were normalised and p-values were calculated using the student t-test. Where there are no p-values data did not reach statistical significance.
Figure 3.15 Effects of low dose wortmannin on insulin- and PMA-induced activation of PI3K-C2α in CHO-IR cells.

CHO-IR cells were serum-starved overnight and then incubated with 100nM wortmannin for 30mins followed by stimulation with either 1μM PMA, for 10mins, or 100nM insulin, for 5mins as indicated. Cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2α specific antisera. PI3K assays were performed on these immunoprecipitates. Results are the means of three experiments carried out in duplicate and are expressed as % of basal (+/ - S.E.M). Basal values were normalised and p-values were calculated using the student t-test where p≤0.01 (**) or where p≤0.05 (*) compared to basal unless otherwise stated. Where there are no p-values data did not reach statistical significance.
Figure 3.16 Effects of Rapamycin on insulin- and PMA-induced activation of PI3K-C2α in CHO-IR cells.

CHO-IR cells were serum-starved overnight and then incubated with 50nM rapamycin for 30mins followed by stimulation with either 1μM PMA, for 10mins, or 100nM insulin, for 5mins as indicated. Cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2α specific antisera. PI3K assays were performed on these immunoprecipitates. Results are the means of four experiments carried out in duplicate and are expressed as % of basal (+/- S.E.M). Basal values were normalised and p-values were calculated using the student t-test where p≤0.01 (**) or where p≤0.05 (*) compared to basal unless otherwise stated. Where there are no p-values data did not reach statistical significance.
Chapter 3 Results

A

\[ \text{gPtdIns-3-P} \quad (\% \text{ basal}) \]

\[ \begin{array}{ccc}
\text{control} & \text{PMA} & \text{PMA+ PD98059} \\
\hline
0 & 100 & 200 \\
\end{array} \]

\[ \star p=0.024 \]

B

\[ \text{GFP-2XFYVEHRS} \quad \text{translocation (\% cells)} \]

\[ \begin{array}{cccc}
0 & 5 & 10 & 15 \\
\hline
10 & 20 & 30 & 40 \\
\end{array} \]

C

\[ \text{GFP-2XFYVEHRS} \quad \text{translocation (\% cells)} \]

\[ \begin{array}{ccc}
\text{Control} & \text{PMA} & \text{PD98059+ PMA} \\
\hline
10 & 50 & 40 \\
\end{array} \]

\[ \star \]
Chapter 3 Results

D

Control

Insulin

PMA

PD98059+PMA
Chapter 3

Results

Figure 3.17 PMA induces PtdIns-3-P formation in L6 cells and is blocked by PD98059.

In collaboration with the research groups of Dr Marco Falasca and Dr Frank Cooke.

(A) L6 cells were labelled with myo-3H-inositol for 24 h and left untreated or pre-treated with 50μM PD98059 for 30 min before stimulation with 1μM PMA for 10 min. Phospholipids were extracted, deacylated and analysed by HPLC. Data are mean ± SEM (n=4) *P <0.05.

(B) GFP-2XFYVE\textsuperscript{Hirn}-transfected L6 cells were serum deprived for 6 h and then left untreated or stimulated with PMA. At the indicated times of stimulation, cells were fixed and analysed by confocal microscopy. Data are mean ± SEM (n=4).

(C) GFP-2XFYVE\textsuperscript{Hirn}-transfected L6 cells were serum deprived for 6 h and left untreated or pre-treated with 50μM PD98059 for 30 min before stimulation with 1μM PMA for 10 min. Cells were fixed and analysed by confocal microscopy. Data are mean ± SEM (n=4). Where there are no p-values data did not reach statistical significance.

(D) GFP-2XFYVE\textsuperscript{Hirn}-transfected L6 cells were serum deprived overnight and then left untreated or pre-treated with 50 μM PD98059 for 30 min. After stimulation with 1 μM PMA for further 10 min cells were fixed and analyzed by confocal microscopy. As a control, untreated cells were incubated with 300 nM insulin for 3 min. Arrows indicate the plasma membrane localization. Bar 10 μm.
Figure 3.18 Effects of PD98059 on the association of PI3K-C2α with different phosphotyrosine proteins in CHO-IR cells.

CHO-IR cells were serum-starved overnight and then pre-incubated with 50μM MEK1 inhibitor PD98059, for 30mins prior to insulin, or PMA stimulation. Cells were then stimulated with 100nM insulin for 5mins, or 1μM PMA, for 10mins. Cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2α specific antisera. Immunoprecipitates were separated by SDS-PAGE and Western blotted with PY99 antiphosphotyrosine antibody.
Figure 3.19 Effects of low dose wortmannin on the association of PI3K-C2α with different phosphotyrosine proteins in CHO-IR cells.

CHO-IR cells were serum-starved overnight and then pre-incubated with 100nM wortmannin for 30mins, prior to insulin, or PMA stimulation. Cells were then stimulated with 100nM insulin for 5mins, or 1μM PMA, for 10mins. Cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2α specific antisera. Immunoprecipitates were separated by SDS-PAGE and Western blotted with PY99 antiphosphotyrosine antibody.
**Chapter 3 Results**

**A**

<table>
<thead>
<tr>
<th></th>
<th>Cell lysates</th>
<th>IP:PI3K-C2α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PMA</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**IB:**

![Cbl-b](image)

**B**

<table>
<thead>
<tr>
<th></th>
<th>Cell lysate</th>
<th>IP:PI3K-C2α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PMA</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**IB:**

![FAK](image)  
![p130 cas](image)
Figure 3.20 Candidates cbl-b, FAK and p130 cas for the 120kDa phosphotyrosine proteins that associate with PI3K-C2α in CHO-IR cells.

CHO-IR cells were serum-starved overnight and then stimulated with either 100nM insulin for 5mins, or 1μM PMA, for 10mins. Cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2α specific antisera. Immunoprecipitates and 50μg cell lysate were separated by SDS-PAGE and Western blotted with (A) cbl-b (B) FAK and p130 cas specific antisera. Similar results were obtained in at least 3 separate experiments.
4. Mechanisms involved in the phosphorylation of class II PI3K

4.1 Summary

This chapter investigates the mechanisms involved in regulating the phosphorylation of PI3K-C2α. We have previously provided evidence that PI3K-C2α becomes phosphorylated after insulin stimulation (Brown, Domin et al., 1999), and we show here that phorbol esters are even more efficient at inducing this in CHO-IR, HEK 293 and L6 myoblast cells. However, we were unable to observe a bandshift indicative of phosphorylation in 3T3-L1 adipocytes suggesting that phosphorylation of PI3K-C2α is cell type specific. By using two strategies such as overnight PMA treatment, and the PKC inhibitor BIM-1, we demonstrate that PKC is directly involved in the phosphorylation of PI3K-C2α in CHO-IR cells. PMA is a powerful activator of the ERK cascade and we demonstrate here by using two highly specific cell permeable inhibitors of MEK, PD98059 and U0126, that the PMA-, but not insulin-induced bandshifts is ERK-dependent in CHO-IR cells. Similarly the same effects were observed in L6 myoblasts after using the inhibitor PD98059. This is the first piece of evidence for the involvement of the ERK cascade in the phosphorylation of a member of the PI3K family. In comparison using the PI3K inhibitor wortmannin, at concentrations that has minimal effects on PI3K-C2α, we found that the insulin-induced, but not the PMA-induced, shift was blocked, suggesting that either class IA or class III PI3K is involved in the phosphorylation of PI3K-C2α.
Evidence has recently been presented for the involvement of JNK in the phosphorylation of PI3K-C2α (Didichenko, Fragoso et al., 2003). The JNK pathway is similar to the ERK cascade, and here we demonstrate that PMA induces the phosphorylation of JNK in CHO-IR and HEK 293 cells but not in 3T3-L1 adipocytes suggesting that phosphorylation of JNK is cell type specific. Furthermore, we demonstrate that JNK phosphorylates PI3K-C2α in vitro. In conclusion we propose that there are different pathways involved in the phosphorylation of PI3K-C2α by PMA and insulin.

4.2 Introduction

Previously it has been shown that class II PI3Ks can be phosphorylated (Didichenko, Fragoso et al., 2003; Didichenko and Thelen 2001; Brown, Domin et al., 1999; Molz, Chen et al., 1996); however, it is not clear what role phosphorylation may play in regulating class II PI3Ks. Both PI3K-C2α (Didichenko and Thelen 2001; Brown, Domin et al., 1999), and PI3K-C2β (Crljen, Volinia et al., 2002; Brown and Shepherd 2001) have been shown to undergo a shift in apparent molecular weight, however, it is not yet clear whether these modifications are required for PI3K-C2α and PI3K-C2β activation. In the case of PI3K-C2α the bandshift appears to be caused by phosphorylation (Didichenko and Thelen 2001; Brown, Domin et al., 1999) while in the case of PI3K-C2β evidence shows that activation involves a calpain-dependent proteolysis step (Crljen, Volinia et al., 2002).

To date there is currently little information on the site(s) or kinase(s) involved in the phosphorylation of PI3K-C2α. In this thesis we were interested in identifying kinases
that are involved in the phosphorylation of PI3K-C2α. It is well known that phorbol esters activate PKC, and that both insulin and phorbol esters activate ERK. As in chapter 3 we have compared the effects of insulin with those of the phorbol ester PMA to try to identify the pathways regulating the phosphorylation of PI3K-C2α. In this present study, we investigated the role of PKC in the phosphorylation of PI3K-C2α by using a two strategies: (1) PKC inhibitors, and (2) chronic treatment of cells with PMA to downregulate PKC (Ishizuka, Cooper et al., 1991). We also tested the involvement of the ERK cascade in the phosphorylation of PI3K-C2α. In this current study we investigated the role of ERK in the PMA-induced phosphorylation of PI3K-C2α by using two different strategies: (1) a highly selective cell permeable chemical inhibitor of MEK (PD98059) that inhibits MEK1 activation and the MAPK cascade (Davies, Reddy et al., 2000; Dudley, Pang et al., 1995); and (2) a cell permeable chemical inhibitor of MEK (UO126) that inhibits MEK1 and MEK2 activation (Davies, Reddy et al., 2000). We also tested whether other classes of PI3K were involved in the phosphorylation of PI3K-C2α. To do this we used the classic PI3K inhibitor wortmannin at concentrations that are known to have little effect on PI3K-C2α activity, but are sufficient to block other isoforms of PI3K (Brown, Domin et al., 1999; Domin, Pages et al., 1997).

To date it is not well understood whether activation and phosphorylation of PI3K-C2α are two independent events, or whether they are directly linked. Recent evidence indicates that phosphorylation of PI3K-C2α regulates proteolytic degradation rather than activation of PI3K-C2α, and that JNK phosphorylates PI3K-C2α (Didichenko, Fragoso et al., 2003). The JNK pathway is homologous to the MAPK/ERK cascade and it is also activated largely by distinct stimuli (Kyriakis and Avruch 1990) including: inflammatory
cytokines, UV light, inhibitors of protein synthesis, and osmotic stress (Kyriakis, Banerjee et al., 1994; Kyriakis and Avruch 1990). Evidence has been shown for the phosphorylation of JNK in response to insulin (D-Mouthon, Eggelpoel et al., 1998; Franklin and Kraft 1995). In this study we investigated whether PMA could phosphorylate JNK in different cell types. We also tested the involvement of the ERK cascade in the PMA, and insulin-induced phosphorylation of JNK, by pre-treating CHO-IR cells with the MEK inhibitor PD98059 followed by PMA stimulation. Furthermore, we also investigated whether JNK recovered from CHO-IR cells could directly phosphorylate PI3K-C2α.

In this present study we demonstrate that PMA as well as insulin induces phosphorylation of PI3K-C2α in a cell type specific manner. Furthermore, we demonstrate that the PMA and insulin-induced bandshifts are PKC-dependent, whereas only the PMA-induced bandshift is ERK-dependent. In comparison we show that the insulin-, but not PMA- induced bandshift, is dependent on other classes of PI3K such as class IA or III PI3K. We also demonstrate that PMA phosphorylates JNK in a cell type-specific manner, whereas phosphorylation of JNK is ERK-dependent. Furthermore, we show that JNK phosphorylates PI3K-C2α in vitro. In conclusion we propose that PMA and insulin regulate phosphorylation of PI3K-C2α by two separate pathways.
Chapter 4

4.3 Results

4.3.1 Effects of insulin and PMA on PI3K-C2\(\alpha\) bandshift and the involvement of PKC

Previously we have shown that insulin treatment of CHO-IR cells causes a change in both activation and phosphorylation of PI3K-C2\(\alpha\) (Brown, Domin et al., 1999), as detected by bandshift. In the previous chapter we investigated the effects of PMA on the activation of PI3K-C2\(\alpha\), and found that PMA-induced the activation of PI3K-C2\(\alpha\) via a pathway independent of PKC. We therefore decided to investigate the effects of PMA on the phosphorylation of PI3K-C2\(\alpha\). Here we find that PMA treatment caused a shift in the apparent molecular weight of the PI3K-C2\(\alpha\) band in CHO-IR and HEK 293 cells, but not in 3T3-L1 adipocytes (Figure 4.1) indicating that phosphorylation in response to PMA is cell type-specific. Insulin also induced phosphorylation of PI3K-C2\(\alpha\) in CHO-IR cells as previously seen (Brown, Domin et al., 1999) (Figure 4.1), but not in 3T3-L1 adipocytes or HEK 293, again suggesting that the insulin-induced phosphorylation of PI3K-C2\(\alpha\) is also cell type specific.

Given the effects of PMA we decided to test whether PKC was directly involved in the phosphorylation of PI3K-C2\(\alpha\) in CHO-IR cells. To do this we used a number of strategies. Firstly we tested the effects of downregulation of PKC by overnight treatment of cells with PMA. In the previous chapter we showed that chronic treatment of CHO-IR cells with PMA down-regulated PKC levels (see chapter 3), but did not reduce the activation of PI3K-C2\(\alpha\) (see chapter 3). Here we show that overnight incubation with PMA, and/or PMA, and/or insulin blocked the PMA- and insulin-induced PI3K-C2\(\alpha\) bandshifts (Figure 4.2). This supports the hypothesis that PKC is directly involved
in the phosphorylation of PI3K-C2α. Total levels of PI3K-C2α were unaffected. Secondly, we tested the effects of the PKC inhibitor BIM-1 on the PMA-induced bandshifts. We found that the PMA-induced shifts were blocked by BIM-1 (Figure 4.3) indicating again that PKC catalytic activity was most likely required for PI3K-C2α phosphorylation.

### 4.3.2 Regulation of PI3K-C2α phosphorylation by ERK and other classes of PI3K

In the previous chapter we investigated the effects of ERK on the activation of PI3K-C2α, and found that PMA-stimulated activity via the ERK pathway. Interestingly, using Scansite (scansite.mit.edu), a programme that predicts phosphorylation sites within a specific sequence, we found that there are two potential ERK1 kinase sites in the N-terminal region of the protein sequence of human PI3K-C2α (accession number NP_002636), and that these sites are conserved between species (accession number of mouse sequence NP_035213).

Given these findings, we decided to test the effects of the ERK cascade on the phosphorylation of PI3K-C2α. Phosphorylation of PI3K-C2α was detected by bandshifts as phosphorylation sites have not been mapped, and thus phospho-specific antibodies are not available. We found that pre-treatment of CHO-IR cells and L6 cells with the MEK inhibitor PD98059 prior to either PMA, or insulin stimulation greatly reduced the PMA-but not insulin-induced bandshift (Figure 4.4). However, effects on the insulin-induced bandshift were not as pronounced as those observed with PMA. The PMA-induced bandshift of PI3K-C2α in CHO-IR cells was also reduced to basal levels by another
MEK inhibitor U0126 further confirming that the ERK pathway was involved in the phosphorylation of PI3K-C2α (Figure 4.5).

To determine whether the ERK cascade was involved in the direct phosphorylation of PI3K-C2α in CHO-IR cells we investigated whether recombinant active ERK could phosphorylate PI3K-C2α in vitro. We immunoprecipitated PI3K-C2α from CHO-IR cells and added recombinant active ERK in vitro, but were unable to demonstrate direct phosphorylation of PI3K-C2α (Figure 4.6). In retrospect, the design of these experiments was flawed as they were performed on PI3K-C2α immunoprecipitates, and the antibody used covers the region containing the putative ERK site potentially hindering access to these sites.

We then tested the involvement of other classes of PI3K on the phosphorylation of PI3K-C2α. To do this we used the classic PI3K inhibitor wortmannin at a concentration that has minimal effects on the activity of PI3K-C2α, but would be sufficient to completely inhibit the activity of all other PI3K isoforms (Domin, Pages et al., 1997; Brown, Domin et al., 1999). In the previous chapter we showed that the insulin, but not PMA, induced activation of PI3K-C2α is blocked by low dose wortmannin. Similarly we found that treatment with low dose wortmannin prior to PMA and insulin stimulation blocked the insulin-induced bandshift, but not the PMA-induced shift (Figure 4.7). This indicated that either class IA or class III PI3K could be involved in the insulin-induced phosphorylation of a PI3K-C2α.
4.3.3 Cell type-specific PMA-dependent phosphorylation of ERK and JNK and phosphorylation of PI3K-C2α by JNK.

The JNK pathway is very similar to the ERK cascade, which we have shown to be involved in the activation (chapter 3) as well as phosphorylation of PI3K-C2α. It has previously been shown that JNK is phosphorylated and activated following insulin stimulation (D-Mouthon, Eggelpoel et al., 1998; Franklin and Kraft 1995). Therefore we decided to test whether PMA could also induce phosphorylation of JNK. In CHO-IR cells we found that ERK was phosphorylated transiently (5 mins) following insulin stimulation, and maintained up to 30 minutes (Figure 4.8). Phosphorylation of ERK-induced by PMA was found to be maximal by 5 minutes, and then sustained for at least 30 minutes (Figure 4.8). We found that JNK was maximally phosphorylated 5 minutes after insulin stimulation, after which phosphorylation was diminished (Figure 4.8), and that JNK was phosphorylated 5 minutes after PMA stimulation, and then sustained for at least 30 minutes. This is similar to that seen with ERK. Total levels of ERK and JNK were confirmed by blotting with total ERK/JNK antisera (Figure 4.8).

To determine whether the ERK cascade was involved in the phosphorylation of JNK we used two highly specific cell permeable inhibitors of MEK, PD98059 and U0126. Pre-incubation of CHO-IR cells with either PD98059 or U0126 prior to stimulation with PMA or insulin blocked the phosphorylation of JNK by insulin and PMA indicating that ERK was required (Figure 4.9).

Furthermore, we investigated whether the phosphorylation of JNK and ERK were cell specific by testing these effects in other cell types such as HEK 293 cells (Figure 4.10) and 3T3-L1 adipocytes (Figure 4.11). We found that insulin stimulation did not
cause any phosphorylation of ERK or JNK in HEK 293 cells or 3T3-L1 adipocytes. Furthermore, we found that PMA stimulation caused phosphorylation of ERK and JNK in HEK 293 (Figure 4.10), whereas only ERK was phosphorylated in 3T3-L1 adipocytes (Figure 4.11). We were unable to show any phosphorylation of JNK in 3T3-L1 adipocytes (Figure 4.11). To confirm that total protein levels were equal we blotted lysates with total ERK and JNK specific antisera (Figures 4.10, 4.11 respectively).

Recently evidence has been presented that indicate that JNK directly phosphorylates PI3K-C2α (Didichenko, Fragoso et al., 2003). As our aim of this study was to identify kinases involved in the phosphorylation of PI3K-C2α, we investigated the effects of JNK on the phosphorylation of PI3K-C2α. To do this we immunoprecipitated JNK from CHO-IR cells stimulated with PMA. We then added to these immunoprecipitates a recombinant N-terminal fragment of PI3K-C2α that was expressed as a GST fusion protein (see methods and materials for GST expressed PI3K-C2α). We found that JNK recovered from PMA-stimulated CHO-IR cells phosphorylates the GST PI3K-C2α fusion protein \textit{in vitro} (Figure 4.12).

4.4 Discussion

It has previously been found that PI3K-C2α is phosphorylated in response to growth factors, but the significance of this is poorly understood and there is currently little information on the site(s) of, or kinase(s) involved. In the previous chapter we found that phorbol esters caused a robust activation of PI3K-C2α, and in this chapter we find that this is associated with phosphorylation of PI3K-C2α as detected by bandshift. It is of note that the bandshift promoted by PMA is consistently greater than that induced by
insulin. This suggested PMA induces a greater degree of phosphorylation. In investigating the kinases responsible for this, the most obvious candidate would be the members of the classical PKC family, as these are potently activated by phorbol esters. Indeed we find evidence for the involvement of PKC as, (1) we find that inhibition of PKC using a PKC specific inhibitor, and (2) downregulation of PKC by overnight chronic PMA treatment (Ishizuka, Cooper et al., 1991), both block the insulin- and PMA-induced phosphorylation of PI3K-C2α. It could well be that PKC directly phosphorylates PI3K-C2α as Scansite identifies two potential PKC consensus phosphorylation sites in PI3K-C2α (Fig 4.13), however, we have not investigated this further.

In chapter 3 we showed that activation of ERK is required for the phorbol ester, but not insulin-induced activation of PI3K-C2α. Here we find PMA, induces phosphorylation of PI3K-C2α. We also show by using two specific inhibitors of MEK, PD98059 and U0126 (Davies, Reddy et al., 2000; Dudley, Pang et al., 1995) that the PMA, but not insulin-induced phosphorylation of PI3K-C2α is ERK-dependent. Interestingly, Scansite predicts two consensus ERK1 phosphorylation sites (Fig 4.13) within the N-terminal region of human PI3K-C2α (accession number NP_002636) (Fig 4.13) with these sites being conserved between human and mouse. We performed preliminary experiments to determine whether ERK could directly phosphorylate PI3K-C2α, and these did not show evidence for such phosphorylation. However, in retrospect the design of these experiments was flawed as they were performed on immunoprecipitates, and the antibody used, covers the region containing the putative ERK sites. Another problem was that basal in vitro phosphorylation of PI3K-C2α was high even in the absence of recombinant active ERK, which made any effects difficult to
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see. This suggested a kinase was co-immunoprecipitating with PI3K-C2α. While this could well be non-specific, it is interesting to note that the PI3K-C2α sequence contains a putative ERK1 binding site (Tanoue and Nishida 2003) (Fig 4.13) suggesting a possible mechanism for direct interaction with ERK. Further studies will be required to determine whether ERK can directly phosphorylate PI3K-C2α, and whether ERK directly associates with it.

While insulin-induced phosphorylation of PI3K-C2α did not require ERK, it was blocked by 100nM wortmannin. This concentration of wortmannin has very little effect on PI3K-C2α kinase activity itself (Brown, Domin et al., 1999; Domin, Pages et al., 1997), therefore suggesting that the effects seen are likely to be due to class IA or class III PI3K, which are both inhibited by wortmannin at this concentration (Brown, Domin et al., 1999; Domin, Pages et al., 1997), and suggests that the effects of insulin are not due to autophosphorylation. Again this is similar to what we observed in chapter 3, where we show that the insulin, but not PMA-induced activation of PI3K-C2α requires either class IA or class III PI3K. It is known that some members of the novel-PKC family are activated by insulin in a wortmannin-sensitive manner, so it is possible these could be responsible for these effects (Leitges, Plomann et al., 2002). The findings above also suggest an explanation for the observation that PMA induces a greater level of phosphorylation of PI3K-C2α than insulin, as insulin is only utilising PKC-dependent phosphorylation while PMA activates two pathways causing phosphorylation of PI3K-C2α. In addition, the Scansite analysis also suggest another possible contributor in that the ERK phosphorylation sites could potentially act as priming sites for phosphorylation by GSK-3 (Fig 4.13). However, insulin causes a very large reduction in GSK-3 activity in
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CHO cells (Murai, Okazaki et al., 1996; Welsh, Foulstone et al., 1994), suggesting such a mechanism is unlikely. PMA, on the other hand, only partially inhibits GSK-3 activity in CHO cells (Murai, Okazaki et al., 1996; Welsh, Foulstone et al., 1994), so there could be significant GSK-3 activity, contributing to phosphorylation of PI3K-C2α in PMA-stimulated cells. The involvement of GSK-3 was not investigated in this thesis, but reasonably selective GSK-3 inhibitors are now available (Martinez, Castro et al., 2002; Coghlan, Culbert et al., 2000) that would allow these investigations to be conducted.

Our studies provide the first evidence for links between PKC and ERK, and phosphorylation of PI3K-C2α, however, the identity of phosphorylation sites in PI3K-C2α, and identity of kinases responsible awaits more detailed investigation using techniques such as mass spectrometry. The potential link between phosphorylation of PI3K-C2α and its activation also remains to be resolved, however, our evidence is consistent with the hypothesis that that phosphorylation is not required for activation. For example in 3T3-L1 adipocytes, we show that insulin induces activation (chapter 3), but does not induce phosphorylation of PI3K-C2α, compared to CHO-IR cells, where PMA induces activation (chapter 3) and phosphorylation of PI3K-C2α. Furthermore, in CHO-IR cells, PMA-induced activation is not blocked by PKC inhibitors, while these inhibitors have a significant effect on phosphorylation induced by PMA. Alternatively, it has also been suggested that phosphorylation of PI3K-C2α is linked to its proteolytic degradation rather than activation of the enzyme (Didichenko, Fragoso et al., 2003). However, these studies found that phosphorylation is dependent on the activation of JNK signalling pathways, but independent of ERK and p38. This would suggest that the PI3K-C2α phosphorylation sites are probably distinct from those involved in the PMA and
insulin effects in this study. However, we do find that JNK is capable of phosphorylating the N-terminal region of PI3K-C2α. In conclusion our studies provide the first evidence for pathways downstream of ERK and PKC being involved in regulating the phosphorylation of PI3K-C2α. Further work will be required to define the physiological significance of these phosphorylation events.
Figure 4.1 Both insulin and PMA stimulation cause a change in the protein mobility of PI3K-C2α in CHO-IR cells, but not 3T3-L1 adipocytes or HEK293 cells.

Cells were serum-starved overnight and then stimulated with 100nM insulin for 5mins or 1μM PMA for 10mins. Cells were lysed, and 50μg of total lysate was subjected to SDS-PAGE and Western blotted with PI3K-C2α-specific antisera. A, CHO-IR cells; B, 3T3-L1 adipocytes and C, HEK 293 cells. Similar results were obtained in at least 3 separate experiments.
Figure 4.2 Chronic concentrations of PMA overnight causes no change in PI3K-C2α protein migration compared to PMA or insulin alone in CHO-IR cells.

CHO-IR cells were serum-starved overnight and then stimulated with 1μM PMA overnight and/or 100nM insulin for 5mins, or 1μM PMA for 10mins. Cells were lysed, and 50μg of cell lysate were subjected to SDS-PAGE and Western blotted with PI3K-C2α specific antisera. Similar results were obtained in at least 3 separate experiments.
**Figure 4.3** The PKC inhibitor BIM-1 inhibits the PMA-stimulated PI3K-C2α bandshift in CHO-IR cells.

CHO-IR cells were serum-starved overnight and then incubated with 5μM PKC inhibitor BIM-1 for 15mins, followed by stimulation with 1μM PMA for 10mins. Cells were lysed, and 50μg cell lysate were subjected to SDS-PAGE and Western blotted with PI3K-C2α specific antisera. Similar results were obtained in at least three experiments.
Figure 4.4 PD98059 inhibits the PMA-stimulated PI3K-C2α bandshift in CHO-IR cells.

CHO-IR cells were serum-starved overnight and then incubated with 50μM MEK1 inhibitor PD98059 for 30mins, followed by stimulation with either 1μM PMA for 10mins, or 100nM insulin for 5mins. Cells were lysed, and 50μg cell lysate were subjected to SDS-PAGE and Western blotted with PI3K-C2α specific antisera. Similar results were obtained in at least three experiments.
Figure 4.5 The MEK inhibitor U0126 inhibits the PMA-stimulated PI3KC2α bandshift in CHO-IR cells.

CHO-IR cells were serum-starved overnight and then incubated with 10μM MEK inhibitor U0126 for 20mins, followed by stimulation with 1μM PMA for 10mins. Cells were lysed, and 50μg cell lysate were subjected to SDS-PAGE and Western blotted with PI3K-C2α specific antisera. Similar results were obtained in at least three experiments.
Figure 4.6 *In vitro* effects of recombinant active ERK on PI3K-C2α kinase activity in CHO-IR cells.

CHO-IR cells were lysed, and lysates were subject to immunoprecipitation using PI3K-C2α-specific antisera. Recombinant active ERK was added to immunoprecipitates *in vitro* as indicated for 15mins. *In vitro* kinase assays were carried out using [$\gamma^{32}$P]ATP and the products were analysed by SDS-PAGE, and autoradiography. Similar results were obtained in at least three experiments.
Figure 4.7 Wortmannin blocks the insulin- but not PMA-induced bandshift in CHO-IR cells.

CHO-IR cells were serum-starved overnight and then incubated with the PI3K inhibitor wortmannin (100nM) for 20mins, followed by stimulation with either 100nM insulin for 5mins, or 1µM PMA for 10mins. Cells were lysed, and 50µg cell lysate were subjected to SDS-PAGE and Western blotted with PI3K-C2α specific antisera. Similar results were obtained in at least three experiments.
Figure 4.8 Phosphorylation of ERK and SAPK/JNK by insulin and PMA in CHO-IR cells.

CHO-IR cells were serum-starved overnight and then stimulated with either 100nM insulin, or 1µM PMA for indicated time-points. Cells were lysed, and 50µg cell lysate were subjected to SDS-PAGE and Western blotted with either phospho or total ERK and SAPK/JNK-specific antisera. Similar results were obtained in at least 3 separate experiments.
Figure 4.9 Phosphorylation of ERK and SAPK/JNK by insulin and PMA is blocked by the MEK inhibitors PD98059 and U0126 in CHO-IR cells.

CHO-IR cells were serum-starved overnight and then incubated with 50µM MEK1 inhibitor PD98059 or 10µM MEK inhibitor U0126 for 20mins, followed by stimulation with either 100nM insulin for 5minutes, or 1µM PMA for 5 minutes. Cells were lysed, and 50µg cell lysate were subjected to SDS-PAGE and Western blotted with either phospho or total ERK and SAPK/JNK-specific antisera. Similar results were obtained in at least 3 separate experiments.
Figure 4.10 Phosphorylation of ERK and SAPK/JNK by PMA and not insulin in HEK293 cells.

HEK 293 cells were serum-starved overnight and stimulated with either 100nM insulin or 1μM PMA for indicated time-points. Cells were lysed, and 50μg cell lysate were subjected to SDS-PAGE and Western blotted with either phospho or total ERK and SAPK/JNK-specific antisera. Similar results were obtained in at least 3 separate experiments.
Figure 4.11 Phosphorylation of ERK and SAPK/JNK by PMA and not insulin in 3T3-L1 adipocytes.

3T3-L1 adipocyte cells were serum-starved overnight and stimulated with either 100nM insulin 1μM PMA for indicated time-points. Cells were lysed, and 50μg cell lysate were subjected to SDS-PAGE and Western blotted with either phospho or total ERK and SAPK/JNK-specific antisera. Similar results were obtained in at least 3 separate experiments.
Figure 4.12 JNK phosphorylates PI3K-C2α \textit{in vitro} in CHO-IR cells.

CHO-IR cells were serum starved overnight and then stimulated with 1μM PMA for 10 minutes. Cells were lysed, and lysates were subjected to immunoprecipitation using JNK-specific antisera. JNK inhibitor (SP600125) 50μM were added \textit{in vitro} to immunoprecipitates where indicated. 5ng of recombinant GST (26kDa) - PI3K-C2α (48kDa) expressed against the N-terminal domain of PI3K-C2α were added to all immunoprecipitates \textit{in vitro}. Control samples were incubated with vehicle (DMSO) only. \textit{In vitro} kinase assays were carried out using [γ-32P]ATP and the products were analysed by SDS-PAGE, and autoradiography. Similar results were obtained in at least three experiments.
<table>
<thead>
<tr>
<th>Predicted group</th>
<th>Predicted phosphorylation sites</th>
<th>Site of phosphorylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine kinase group (Y_Kin)</td>
<td>EGFR Kinase</td>
<td>Y186</td>
</tr>
<tr>
<td>Src homology 3 group (SH3)</td>
<td>Cortactin SH3</td>
<td>P1094</td>
</tr>
<tr>
<td>Basophilic serine/threonine kinase group (Baso_ST_Kin)</td>
<td>two PKCa,β and γ</td>
<td>S1615 and S60</td>
</tr>
<tr>
<td>Acidophilic serine/threonine kinase group (Acid_ST_Kin)</td>
<td>two GSK-3 Kinase</td>
<td>T120 and S197</td>
</tr>
<tr>
<td>Proline-dependent serine/threonine kinase group (Pro_ST_Kin)</td>
<td>p38 MAPK and two Erk1 Kinase</td>
<td>T120, T201 and S124</td>
</tr>
<tr>
<td>Kinase binding site group (Kin_bind)</td>
<td>Erk1 Binding site</td>
<td>P199</td>
</tr>
<tr>
<td>Phosphotyrosine binding group (PTB)</td>
<td>Shc PTB site</td>
<td>Y406</td>
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Fig 4.13 Predicted phosphorylation sites found in the protein sequence of PI3K-C2α (accession number NP_002636) from *Homo sapiens* using the Scansite website (scansite.mit.edu).
5. New evidence for the regulation of class II PI3K by cytokines

5.1 Summary

The class II PI3Ks are known to be activated by growth factors and chemokines, but to date there are no reports of cytokine mediated regulation. Here we have investigated the effects of the cytokines TNFα and leptin on the activation of PI3K-C2α. We have found that TNFα and leptin both induce the activation of PI3K-C2α in J774.2 macrophages, and CHO-IR cells respectively. We also demonstrate that the actions of both cytokines are blocked by PD98059, an inhibitor of MEK activation. These findings indicate that the cytokines activate PI3K-C2α, and do so by a mechanism that requires activation of the ERK pathway.

We have previously shown that insulin stimulates both phosphorylation of PI3K-C2α, and association with tyrosine phosphorylated proteins (Brown, Domin et al., 1999). Here we show that leptin stimulation of J774.2 macrophages causes neither a phosphorylation-induced bandshift, or the association of PI3K-C2α with tyrosine phosphorylated proteins. In comparison we find that TNFα induces the association of PI3K-C2α with a tyrosine phosphorylated protein of 120kDa in CHO-IR cells. In chapter 3 we have provided evidence that the 120kDa phosphorylated band contains the focal adhesion proteins, FAK and p130cas. In the case of leptin we have compared the activation of PI3K-C2α with the activation of class I PI3K. We find that different isoforms of PI3K are recruited to phosphotyrosine complexes after leptin stimulation. In
conclusion we show that two different hormones, acting through two different classes of cytokine receptor in two different cell types, activate PI3K-C2α in an ERK-dependent manner.

5.2 Introduction

The effects of a wide range of stimuli, including growth factors (Brown, Domin et al., 1999; Urso, Brown et al., 1999), chemokines (Turner, Domin et al., 1998) and integrin receptors (Zhang, Banfic et al., 1998) on the activation of PI3K-C2α have been investigated. To date there is no evidence for the involvement of cytokines in the regulation of PI3K-C2α. In this present study, we have compared the effects of the cytokines leptin and TNFα on the regulation of PI3K-C2α, to try to identify pathways upstream of PI3K-C2α.

Leptin and TNFα are two pro-inflammatory cytokines (Kudo, Fujikawa et al., 2002; Loffreda, Yang et al., 1998), with elevated levels of both being found in obese individuals (Hotamisligil 1999; Sethi and Hotamisligil 1999; Hotamisligil, Shargill et al., 1993). Leptin and TNFα exhibit different mechanisms of signal transduction. Leptin is an adipocyte-secreted hormone that acts in the hypothalamus to regulate weight control. Leptin has very low sequence homology to other genes, but the 3D structure is very similar to the IL-6 family of cytokines (Bauman, Morella et al., 1996). Leptin signals primarily through the long form of the receptor (ObRb) (Tartaglia 1997), which has signalling capabilities of IL-6 class of cytokine receptors (Bauman, Morella et al., 1996). This class of cytokines induces homodimerisation of their cognate receptors, resulting in activation of associated Janus (JAK) kinases, and subsequent tyrosine phosphorylation of...
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signal transducer and activator of transcription 3 (STAT3) (Heinrich, Behrmann et al., 1998; White, Kutropatwinski et al., 1997). We have previously shown that macrophages are target tissues for leptin signalling (O'Rourke, Yeamen et al., 2001). Thus, we have used these cells in this study. We have also shown that ObRb is expressed to high levels in macrophages (O'Rourke, Yeamen et al., 2001), and is capable of activating class I PI3K and ERK (O'Rourke, Yeamen et al., 2001; Sanchez-Alvarez, Gobema et al., 1999).

TNFα exerts its effects by binding to the TNF receptor 1 (TNFR1) and TNFR2, which are expressed in virtually all types of cells (Chen and Goeddel 2002). TNFR1 has been found to initiate the majority of TNF’s biological activities. The binding of TNF to TNFR1 leads to recruitment of TRADD (TNFR associated death domain) and various other adaptor proteins responsible for initiating signalling events, leading to the activation of two major transcription factors: nuclear factor κB (NF-κB), and c-jun (MacEwan 2002). Other pathways are also activated in response to TNFα, for example, it has been shown, using both MEK and PI3K inhibitors, that insulin and TNFα regulate the expression of Foxc2 via a PI3K and ERK 1/2-dependent pathway in 3T3-L1 adipocytes (Gronning, Cedergberg et al., 2002).

In this current study we have investigated the effects of leptin and TNFα on the activation of PI3K-C2α, as well as the effects of leptin on the phosphorylation of PI3K-C2α. We also investigated the role of ERK, a downstream effector of PI3K on the leptin and TNFα-induced activation of PI3K-C2α. To do this we used a highly-selective cell-permeable chemical inhibitor of MEK (PD98059), that inhibits MEK1 activation and the MAPK cascade (Davies, Reddy et al., 2000; Dudley, Pang et al., 1995). Furthermore, we investigated the effects of class I PI3K by testing the effects of leptin on the recruitment
of different isoforms of class I PI3K in comparison to the recruitment of PI3K-C2α. In addition to being able to measure changes in activity in response to cytokines such as leptin and TNFα, we have previously observed that insulin induces association of PI3K-C2α with a 160kDa tyrosine phosphorylated protein (Brown, Domin et al., 1999). Such an association is very likely to have an effect on PI3K-C2α function, so we have investigated the effects of leptin and TNFα on the association of PI3K-C2α with tyrosine phosphorylated proteins in order to determine which signalling pathways might be involved in regulating any associations identified.

In this present study, we show that the cytokines leptin and TNFα both induce the activation of PI3K-C2α, and that this activation is ERK-dependent. Furthermore we demonstrate that TNFα, but not leptin, induces the association of PI3K-C2α with a 120kDa tyrosine-phosphorylated band. In chapter 3 we found this band contains members of the focal adhesion complex, FAK and p130cas. Such data is the first evidence that cytokines regulate PI3K-C2α via the ERK cascade, and provide new insights into the mechanisms by which these cytokines regulate cellular function.

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5.3.1 Leptin activation of PI3K-C2α

To date there is no evidence for the involvement of cytokines in the activation of PI3K-C2α; therefore, we decided to look at the effects of the cytokine leptin in J774.2 macrophages, which is a leptin-responsive cell-type. We have previously shown that 2nM leptin is maximally effective at activating leptin signalling pathways in J774.2 macrophages (O'Rourke, Yeamen et al., 2001), and we find here that this concentration of
leptin significantly induces the activation of PI3K-C2α (Figure 5.1). This activation was maximal after 15 to 20 minutes of leptin stimulation, where an approximate four-fold increase in PI3K-C2α activity was observed. Stimulation with leptin did not cause any detectable shift in electrophoretic mobility of PI3K-C2α (Figure 5.2) in J774.2 macrophages, suggesting that leptin may not induce the phosphorylation of PI3K-C2α.

We have previously demonstrated that PI3K-C2α associates with a 160kDa tyrosine phosphorylated protein after insulin stimulation (Brown, Domin et al., 1999) (see chapter 3). To determine if the same effects occur following leptin stimulation we immunoprecipitated PI3K-C2α from in J774.2 macrophage cells stimulated with leptin, and then western blotted with anti-phosphotyrosine antibody. We found that a protein with a similar molecular weight to PI3K-C2α was tyrosine phosphorylated upon increased leptin stimulation (Figure 5.3). Western blotting of immunoprecipitates with PI3K-C2α-specific antisera confirmed that the protein was in fact PI3K-C2α (Figure 5.3) suggesting that PI3K-C2α is tyrosine phosphorylated.

5.3.2 Leptin regulation of PI3K-C2α is ERK-dependent

In the previous chapter we found that the PMA activation of PI3K-C2α was ERK-dependent; therefore, we decided to test whether leptin activation of PI3K-C2α was also ERK-dependent in J774.2 macrophages. Using the MEK inhibitor PD98059 at concentrations that we have previously shown to block the activation of ERK's in J774.2 macrophages (O'Rourke and Shepherd 2002), we found that the leptin-induced activation of PI3K-C2α was reduced significantly by pre-treatment of the cells with PD98059
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(Figure 5.4). This suggested that the ERK cascade was necessary for the leptin-induced activation of PI3K-C2α.

5.3.3 Leptin stimulation causes recruitment of PI3K isoforms to phosphotyrosine complexes.

To determine whether class I PI3K was involved in the regulation of PI3K-C2α by leptin we investigated the recruitment of PI3K isoforms to tyrosine phosphorylated signalling complexes. To do this anti-phospho tyrosine immunoprecipitates were obtained from J774.2 macrophages stimulated with leptin, and then western blotted with antibodies specific for the different isoforms of PI3K (Figure 5.5). Western blotting with anti-PI3K-C2α demonstrated that it is recruited to such complexes after 10 minutes leptin stimulation. Both p85α and p85β showed a dramatic increase in recruitment after 15 minutes leptin stimulation, as did p110β. No increase was observed for p110δ upon stimulation with leptin. In unstimulated cells, basal recruitment of this isoform to phosphotyrosine complexes was found to be very high (Figure 5.5). To test this we immunoprecipitated p110δ from cells stimulated with both insulin and leptin and western blotted with anti-phosphotyrosine antisera (Figure 5.6). We found that p110δ itself was tyrosine phosphorylated. To further confirm that the phosphorylated protein was p110δ, we western blotted back with p110δ specific antisera. The effects we see are most likely due to p110δ being constitutively tyrosine phosphorylated.
Results

5.3.4 TNFα activates PI3K-C2α through a ERK-dependent mechanism

As well as investigating the effects of leptin we tested the effects of another cytokine, TNFα which utilises a different signalling mechanism. Using concentrations of TNFα (25ng/ml) that have previously been shown to activate PI3K-mediated cascades (Gronning, Cedergberg et al., 2002), we found that stimulation of CHO-IR cells with TNFα induced the activation of PI3K-C2α (Figure 5.7). This activation was maximal after 10 minutes of stimulation, where an approximate 2-3-fold increase in PI3K-C2α activity was observed. We also found that stimulation with TNFα for approximately 5-10 minutes resulted in transient phosphorylation of ERK1/ERK2 (Figure 5.7). To test the involvement of ERK in the TNFα-induced activation of PI3K-C2α in CHO-IR cells we used the MEK inhibitor PD98059. We found that the TNFα-induced activation of PI3K-C2α was reduced to basal levels after PD98059 treatment (Figure 5.8) suggesting that activation of ERK is required in this regulation. Western blotting with phospho-specific ERK 1/2 antibodies of cell lysates from cells pre-incubated with PD98059 and then stimulated with TNFα confirmed that ERK activation was blocked hence the inhibitor PD98059 was working (Figure 5.8).

Furthermore, stimulation of J774.2 macrophages with 25ng/ml TNFα significantly induced the activation of PI3K-C2α (Figure 5.9). This activation was maximal after 10 minutes of stimulation, where an approximate 2-2.5-fold increase in PI3K-C2α activity was observed. Stimulation with TNFα resulted in very little phosphorylation of ERK1/ERK2 (Figure 5.9) due to such a high basal level of phosphorylation, something observed previously with leptin in these cells (O'Rourke and Shepherd 2002) and now with TNFα.
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As with leptin we decided to look at the effects of TNFα on the association of PI3K-C2α with tyrosine phosphorylated proteins. To do this we immunoprecipitated PI3K-C2α from CHO-IR cell lysates stimulated with TNFα or insulin, and then western blotted with anti-phosphotyrosine antisera (Figure 5.10). Insulin stimulation led to the increased association of PI3K-C2α with two phosphotyrosine proteins one of 160kDa which we have previously reported (Brown, Domin et al., 1999) and another of 106kDa (Figure 5.10). But what was interesting is that TNFα stimulation led to the increased association of PI3K-C2α with a phosphotyrosine protein of 120kDa (Figure 5.10) similar to the effects we observed upon PMA stimulation in chapter 3.

5.4 Discussion

At the outset of the current studies there were no reports to show that cytokines were involved in the regulation of PI3K-C2α. We therefore decided to investigate the effects of two different cytokines, leptin and TNFα, on the activation of PI3K-C2α. Leptin and TNFα are two cytokines which act through receptors that use different signalling mechanisms, but both have previously been shown to activate class I PI3K activity (Gronning, Cedergberg et al., 2002; M-Romero and Sanchez-Margalet 2001; Bjorbaek, Uotani et al., 1997; Kellerer, Koch et al., 1997). Here, we show for the first time that PI3K-C2α is activated by these cytokines. We show that leptin stimulation of J774.2 macrophages induces maximal PI3K-C2α activation after 20 minutes, which correlates well with previously reported data on leptin signalling in J774.2 macrophages, where activation of ObRb, JAK-2, STAT3 and ERK all reach maximal levels after 15-20
minutes of leptin stimulation (O'Rourke and Shepherd 2002; O'Rourke, Yeamen et al., 2001). In comparison, leptin stimulation of PI3K-C2α was different to that seen with insulin in chapter 3. Maximal activation of PI3K-C2α by leptin was observed after 20 minutes stimulation, compared to insulin where maximum activation was observed after 5 minutes stimulation. In addition, there was a small change in PI3K-C2α activity after 5-10 minutes leptin stimulation in every assay performed. Interestingly, ERK1/2 phosphorylation has also been shown to be transiently reduced by leptin around this timepoint (O'Rourke and Shepherd 2002; Brink, O'Toole et al., 2000). We therefore decided to use the highly-specific cell-permeable MEK inhibitor, PD98059 (Davies, Reddy et al., 2000; Dudley, Pang et al., 1995). We show that the leptin-induced activation of PI3K-C2α, unlike insulin (see chapter 3), was largely blocked by PD98059, suggesting that leptin can regulate PI3K-C2α activation via ERK.

Our results further show that TNFα stimulation induces maximal PI3K-C2α activation after 10 minutes in both J774.2 macrophages and CHO-IR cells. In comparison, TNFα-induced activation of PI3K-C2α was different to that seen with insulin in chapter 3. Maximal activation of PI3K-C2α by TNFα was observed after 10 minutes stimulation, compared to insulin where maximal activation was observed after 5 minutes stimulation. Further the TNFα-induced activation of PI3K-C2α was completely blocked by PD98059 in CHO-IR cells, suggesting that activation is entirely ERK-dependent in response to this factor. Previous reports have shown that growth factors (Brown, Domin et al., 1999) and chemokines (Turner, Domin et al., 1998) activate PI3K-C2α; however, the signalling mechanisms leading to this activation of PI3K-C2α have not been elucidated. The ERK-dependent pathway demonstrated here is the first time a
signalling pathway leading to the class II PI3Ks has been identified. Interestingly, TNF\(\alpha\) induced the association of PI3K-C2\(\alpha\) with a 120kDa tyrosine phosphorylated protein in CHO-IR cells similar to that seen after PMA stimulation in chapter 3. We have provided evidence in chapter 3 that this tyrosine phosphorylated band contains members of the focal adhesion proteins FAK and p130cas that are involved in cellular adhesion (see chapter 3).

Our data indicates that both leptin and TNF\(\alpha\) activate PI3K-C2\(\alpha\) by a mechanism that is distinct from that used by insulin, but similar to that used by PMA (see final discussion). In summary our data shows that the cytokines leptin and TNF\(\alpha\) are capable of activating class-II PI3K, and that this activation could be dependent upon JAK2 at least for the leptin regulation of PI3K-C2\(\alpha\). Future investigations would be required to confirm this. In conclusion our findings represent the first evidence that PI3K-C2\(\alpha\) is activated downstream of cytokine receptors and suggest a novel role for PI3K-C2\(\alpha\) in cytokine signalling cascades. Our findings also provide evidence for the involvement of the ERK cascade in the cytokine-mediated activation of PI3K-C2\(\alpha\)
Figure 5.1 Leptin induced activation of PI3K-C2α in J774.2 macrophages.

J774.2 macrophage cells were serum-starved overnight and then stimulated with 2nM leptin for indicated times. Cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2α specific antisera. PI3K assays were performed on these immunoprecipitates. Results are the means of four experiments carried out in quadruplicates. Results are expressed as % of basal (+/ - S.E.M). Basal values were normalised and p-values were calculated using the student t-test where p≤0.01 (**) or where p≤0.05 (*) compared to basal unless otherwise stated. Where there are no p-values data did not reach statistical significance.
Chapter 5 Results

Leptin stimulation (mins)

0  5  10  20  30

PI3K-C2α

Figure 5.2 Leptin stimulation does not cause a bandshift with PI3K-C2α in J774.2 macrophages.

J774.2 macrophage cells were serum-starved overnight and then stimulated with 2nM leptin for indicated timepoints. Cells were lysed, and 50µg cell lysate were subjected to SDS-PAGE and Western blotted with PI3K-C2α specific antisera. Similar results were obtained in at least three experiments.
Figure 5.3 Leptin stimulation in J774.2 macrophages causes tyrosine phosphorylation of PI3K-C2α.

J774.2 macrophage cells were serum-starved overnight and then stimulated with 2nM leptin for indicated time-points. Cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2α-specific antisera. Immunoprecipitates were separated by SDS-PAGE and Western blotted as indicated, with either anti-PI3K-C2α or anti-phosphotyrosine (pTyr) antibodies. Similar results were obtained in at least three experiments.
Figure 5.4 PD98059 blocks leptin induced activation of PI3K-C2α activity in J774.2 macrophages.

J774.2 macrophage cells were serum-starved overnight and then incubated with 50μM PD98059 prior to addition of 2nM leptin for 20 minutes. Cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2α specific antisera. PI3K assays were performed on these immunoprecipitates. Results are the means of three experiments carried out in duplicates. Results are expressed as % of basal (+/- S.E.M). Basal values were normalised and p-values were calculated using the student t-test where \( p \leq 0.01 \) (**) or where \( p \leq 0.05 \) (*) compared to basal unless otherwise stated. Where there are no p-values data did not reach statistical significance.
Figure 5.5 Leptin and insulin cause recruitment of Class I and Class II PI3K isoforms to phosphotyrosine complexes in J774.2 macrophages.

J774.2 macrophage cells were serum-starved overnight and then stimulated with 2nM leptin for indicated timepoints. Cells were lysed, and lysates were subjected to immunoprecipitation using anti-phosphotyrosine (p-Tyr), specific antisera. Immunoprecipitates were separated by SDS-PAGE and western blotted as indicated, with antibodies raised against PI3K-C2α, p85, p110β and p110δ. Similar results were obtained in at least three experiments.
Figure 5.6 Leptin stimulation in J774.2 macrophages causes tyrosine phosphorylation of p110δ.

J774.2 macrophage cells were serum-starved overnight and then stimulated with 2nM leptin for 15 minutes or 100nM insulin for 10 minutes. Cells were lysed, and lysates were subjected to immunoprecipitation using p110δ specific antisera. Immunoprecipitates were separated by SDS-PAGE and Western blotted as indicated, with either anti-p110δ or anti-phosphotyrosine (pTyr) antibodies. Similar results were obtained in at least three experiments.
Chapter 5 Results

A

PI3K activity (% of basal)

Basal 5' 10' 15' 30'

TNFα stimulation (mins)

B

TNFα 25ng/ml (mins) 0 5 10 15 30

p-ERK

ERK
Figure 5.7 TNFα stimulation causes phosphorylation of ERK and activation of PI3K-C2α in CHO-IR cells.

CHO-IR cells were serum-starved overnight and then stimulated with 25ng/ml recombinant TNFα for indicated times. Cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2α specific antisera. PI3K assays were performed on these immunoprecipitates (A). Results are the means of three experiments carried out in duplicates. Results are expressed as % of basal (+/ - S.E.M). Basal values were normalised, and p-values were calculated using the student t-test where p≤0.01 (**) or where p≤0.05 (*) compared to basal unless otherwise stated. Where there are no p-values data did not reach statistical significance. Cell lysates (50µg) were subjected to SDS-PAGE and Western blotted with either phospho or total ERK-specific antisera (B). Similar results were obtained in at least three experiments.
Chapter 5 Results

PI3K activity (% of basal)

A

Treatment of cells

B

PD98059  -  -  +  +

TNFα  -  +  -  +

p-ERK

ERK

179
Figure 5.8 TNFα-induced activation of PI3K-C2α is ERK-dependent in CHO-IR cells.

CHO-IR cells were serum-starved overnight and then stimulated with 50μM PD98059 prior to 25ng/ml recombinant TNFα for 10 minutes. Cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2α specific antisera. PI3K assays were performed on these immunoprecipitates (A). Results are the means of four experiments carried out in duplicates. Results are expressed as % of basal (+/ - S.E.M). Basal values were normalised, and p-values were calculated using the student t-test where $p \leq 0.01$ (**), or where $p \leq 0.05$ (*) compared to basal unless otherwise stated. Where there are no p-values data did not reach statistical significance.

Cell lysates (50μg) were subjected to SDS-PAGE and Western blotted with either phospho or total ERK-specific antisera (B). Similar results were obtained for at least three experiments.
Chapter 5 Results

A

PI3K activity (% of basal)

Basal 5' 10' 15' 30'

TNFα stimulation time (mins)

B

TNFα 25ng/ml (mins)

0 5 10 15 30

p-ERK

ERK
Figure 5.9 TNFα stimulation causes phosphorylation of ERK and activation of PI3K-C2α in J774.2 macrophage cells.

J774.2 macrophage cells were serum-starved overnight and then stimulated with 25ng/ml recombinant TNFα for indicated times. Cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2α specific antisera. PI3-Kinase assays were performed on these immunoprecipitates (A). Results are the means of three experiments carried out in duplicates. Results are expressed as % of basal (+/- S.E.M). Basal values were normalised, and p-values were calculated using the student t-test where p≤0.01 (**) or where p≤0.05 (*) compared to basal unless otherwise stated. Where there are no p-values data did not reach statistical significance.

Cell lysates (50μg) were subjected to SDS-PAGE and Western blotted with either phospho or total ERK-specific antisera (B). Similar results were obtained in at least three experiments.
Figure 5.10 Effects of TNFα on the association of PI3K-C2α with different phosphotyrosine proteins in CHO-IR cells.

CHO-IR cells were serum-starved overnight and then stimulated with either 100nM insulin for 5 minutes, or 25ng/ml TNFα for 10 minutes. Cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2α-specific antisera. Immunoprecipitates were separated by SDS-PAGE and Western blotted with PY99 anti-phosphotyrosine antibody.
Chapter 6 Results

6. Regulation of class IA and class II PI3K in rat soleus muscle

6.1 Summary

This chapter investigates the mechanisms involved in regulating the activity of PI3K-C2α in rat muscle. Recently one of our collaborators (Dr. Jorgen Jensen) observed that adrenaline potentiates insulin’s effect on the activation of PKB in a wortmannin-sensitive manner, suggesting that PI3K could be regulated by adrenaline. We demonstrate that in muscle, insulin induces the recruitment of class IA p85 adapter subunit to tyrosine phosphorylated complexes, whereas adrenaline alone had no effect on either. Adrenaline however modulated insulin’s effect on PI3K in a biphasic manner with a potentiation of insulin’s effects observed at early timepoints (<10min), but an attenuation of insulin’s effects at later timepoints (>10min). In comparison both insulin and adrenaline induced the activation of class II PI3K (PI3K-C2α) in rat soleus muscle, whereas only insulin induced recruitment of PI3K-C2α to tyrosine phosphorylated complexes. These data suggest that different pathways are involved in the regulation of class IA and class II PI3K. Class II PI3K has recently been shown to lie upstream of PKB (Arcaro, Khanzada et al., 2002), and we demonstrate here that insulin stimulation of rat soleus muscle induces phosphorylation of PKB. We further decided to investigate the effects of the cAMP analogue (8’-CPT’-cAMP) on the activation of PI3K-C2α. We demonstrate that cAMP analogues induce the activation of PI3K-C2α, and that this activation is dependent
6.2 Introduction

Muscle is a major site of insulin-mediated glucose disposal and activation of the class IA PI3K appears to be required for insulin’s effects on glucose metabolism (Lund, Holman et al., 1995; Yeh, Gulve et al., 1995). Adrenaline and insulin are two of the most important regulators of glucose metabolic regulation (Shepherd and Kahn 1999; Clutter, Rizza et al., 1988); and have different metabolic outputs. Adrenaline binds to β-adrenergic receptors, thus producing cAMP which then leads to the activation of PKA and the subsequent breakdown of glycogen (Meyer, Heilmeyer et al., 1970). Insulin’s effect on metabolism requires PI3K-dependent activation of PKB, for example, stimulation of glycogen synthase (Shepherd, Nave et al., 1995), Glut 4 translocation (Hajduch, Alessi et al., 1998), protein synthesis (Nave, Ouwens et al., 1999) and gene expression (Vanhaesebroeck and Alessi 2000).

Here in this study we have decided to investigate the effects of the hormone adrenaline on insulin-stimulated activation of class IA and class II PI3K (PI3K-C2α). In these studies we have compared the effects of insulin and adrenaline on both class IA and class II PI3K in the hope that this comparison would lead to the identification of pathways regulating the activation of PI3K-C2α in rat soleus muscle. Previously we have shown that insulin acutely activates both class IA and class II PI3K enzymes in muscle (Soos, Jensen et al., 2001), thus providing the first piece of evidence that class II PI3K
could be involved in insulin signalling pathways in muscle (Soos, Jensen et al., 2001). Recently one of our collaborators (Prof. Jorgen Jenson) has observed that adrenaline potentiates insulin’s effect on the activation of PKB in a wortmannin-sensitive manner, suggesting that PI3K could be regulated by adrenaline. Reports have also shown that class II PI3K can activate PKB (Arcaro, Khanzada et al., 2002); therefore, we decided to test whether insulin and adrenaline could phosphorylate PKB in rat soleus muscle. It is known that cAMP-elevating agents activate PKB in many cell types (Moule, Welsh et al., 1997; Sable, Filippa et al., 1997), and in others decrease PKB activation (Mei, Qiao et al., 2002). A clue to the reason for this came from the finding that in cells where cAMP activates PKB it is generally independent of PKA activation (Meroni, Riera et al., 2002), and in fact, PKA inhibits PKB activation in some cell types (Mei, Qiao et al., 2002). This indicated that the effects were mediated by another cAMP target such as the GTPase exchange factor (GEF) Epac. Epac GEF activity is directly activated by cAMP (Rooij, Zwartkruis et al., 1998). We decided to investigate the effects of the cAMP analogue (8’-CPT’-cAMP) on the activation of PI3K-C2α in a cell type (CHO-IR cells) other than muscle.

In addition to directly increasing class IA PI3K activity, its function in cells can also be regulated by recruitment to appropriate tyrosine phosphorylated complexes via the SH2 domain of p85. To test whether adrenaline and insulin regulated this process, we assessed the recruitment of p85 as well as that of PI3K-C2α to tyrosine phosphorylated complexes using anti-phosphotyrosine antibody immunoprecipitates. Further in collaboration with the research group of Dr. Jorgen Jensen, we investigated the effects of insulin and adrenaline on the regulation of class IA and class II PI3K by stimulating
muscle extracts with either insulin or adrenaline, and then measuring the effects by lipid kinase assays. In conclusion, we propose that insulin and adrenaline regulate class IA and class II PI3K in rat soleus muscle through different pathways.

6.3 Results

6.3.1 Effects of insulin and adrenaline on the activation of class IA and PI3K-C2α in rat soleus muscle

We decided to investigate the effects of both adrenaline and insulin on the activation of class IA and PI3K-C2α. We found that stimulation of rat soleus muscle with insulin for 30 minutes induced the activation of class IA PI3K almost 2.4-fold as measured in p85 immunoprecipitates (Figure 6.1). Insulin's effects were not blocked by adrenaline while adrenaline alone had no effect (Figure 6.1). In comparison we found that stimulation of muscle with insulin and adrenaline for 30 minutes induced PI3K-C2α activation 2.5-fold and 3.5 fold respectively. Treatment with both insulin and adrenaline also induced activation of PI3K-C2α approximately 3 fold (Figure 6.2). These data demonstrate that adrenaline and insulin induces the activation of PI3K-C2α, whereas only insulin induces the activation of class IA PI3K suggesting that a different mechanism be involved. The results followed the same trend in all three experiments carried out, however the data did not reach statistical significance.
6.3.2 Insulin stimulates PKB phosphorylation and cAMP elevating agents cause activation of PI3K-C2α via PKA

The effects of adrenaline are presumably mediated by cAMP so we decided to test whether cAMP could directly activate PI3K-C2α in a cell type other than muscle. We show that the cAMP analogue (8'-CPT'-cAMP) induces the activation of PI3K-C2α in CHO-IR cells (Figure 6.3). Furthermore, using the PKA inhibitor H-89, we found that this cAMP induced activation of PI3K-C2α is blocked suggesting that the cAMP effect be mediated via PKA (Figure 6.3).

During the course of these studies one of our collaborators (Dr. Jorgen Jensen) observed that adrenaline potentiates insulin’s effect on the activation of PKB in a wortmannin-sensitive manner, suggesting that PI3K could be regulated by adrenaline. Furthermore, reports have shown that class II PI3K can activate PKB (Arcaro, Khanzada et al., 2002); therefore, we decided to test whether insulin and adrenaline could induce phosphorylation of PKB in our rat soleus muscle system. We show that insulin stimulation of rat soleus muscle induces phosphorylation of PKB on residue threonine 308 (Figure 6.4). The effects seem to be synergistic as treatment of rat soleus muscle with both insulin and adrenaline also phosphorylates PKB threonine 308 (Figure 6.4). The results followed the same trend in all three experiments carried out, however the data did not reach statistical significance.
6.3.3 Recruitment of class IA and PI3K-C2α to phospho-tyrosine complexes after insulin and adrenaline stimulation in rat soleus muscle

In addition to directly increasing intrinsic activity of class IA PI3K, its function in cells can also be regulated by recruitment to appropriate tyrosine phosphorylated complexes via the SH2 of p85. To test whether adrenaline and insulin regulated this process we assessed the recruitment of p85 and PI3K-C2α to tyrosine phosphorylated complexes using anti-phosphotyrosine antibody immunoprecipitates. We found that treatment of muscle with insulin increased recruitment of class IA PI3K to phosphotyrosine complexes, with maximal recruitment 2.5 fold after 15 minutes stimulation (Figure 6.5). Adrenaline alone did not cause any change in the recruitment of class I PI3K. However, treatment with both insulin and adrenaline increased recruitment of class I PI3K to phospho-tyrosine complexes with time, with maximal recruitment 4 fold after 5 minutes stimulation. Stimulation then steeply decreased with time, after 15 minutes-stimulation, recruitment of PI3K reached basal levels (Figure 6.5).

PI3K-C2α has also been found to associate with tyrosine phosphorylated proteins (see chapter 3). We found that treatment of muscle with insulin for 30 minutes increased the recruitment of PI3K-C2α to phosphotyrosine complexes by approximately 1.5 fold (Figure 6.6). However, treatment with adrenaline, or adrenaline and insulin for 30 minutes did not cause any change in the recruitment of PI3K-C2α (Figure 6.6). Furthermore, we cannot rule out that these effects seen are due to tyrosine phosphorylation of PI3K-C2α or recruitment to phosphotyrosine complexes. The results followed the same trend in all three experiments carried out, however the data did not reach statistical significance.
6.4 Discussion

A number of signalling pathways act via increasing cellular levels of cAMP but it was not known whether such increases would regulate PI3K-C2α. However, there are reports that in some cell types that cAMP stimulates pathways that lead to the activation of PKB (Moule, Welsh et al., 1997; Sable, Filippa et al., 1997). Further, our collaborator, Dr Jorgen Jensen, has observed that adrenaline potentiates the insulin-induced activation of PKB. Adrenaline acts via the β-adrenergic receptor to increase cellular levels of cAMP, and this pathway is particularly important in muscle, which expresses high levels of the β-adrenergic receptors. Current evidence indicates that PI3K activity is required for PKB activation, with both the class-I and class-II PI3Ks having been implicated (Arcaro, Khanzada et al., 2002). However, the effects of cAMP on PI3K activity have not been fully explored. Here we investigated the effects of adrenaline and cAMP on both class I and class-II PI3K.

It has previously been reported that insulin activates class IA and class II PI3K (PI3K-C2α) in rat muscle soleus (Soos, Jensen et al., 2001). Here in this chapter we show that adrenaline is even more efficient than insulin at inducing the activation of PI3K-C2α. At the time points studied only insulin regulated the intrinsic activity of class IA PI3K. Furthermore, we demonstrate that class IA and class II PI3K are both recruited to phosphotyrosine complexes in soleus muscle upon insulin treatment. The recruitment of class IA PI3K to tyrosine phosphorylated proteins such as IRS-1 is generally accepted as the major mechanism by which it is activated. It is therefore of interest that adrenaline modulated insulin’s effect on recruitment of class IA PI3K to tyrosine phosphorylated proteins.
proteins in a biphasic manner, with a potentiation of insulin’s effects observed at early
timepoints (<10min), but an attenuation of insulins’s effects seen at later time points.
The complex interaction between insulin and adrenaline is very interesting and has not
previously been observed.

As PI3K-C2α was activated by adrenaline, we went on to test whether cAMP
could activate the enzyme in other cell types. We found that the PKA inhibitor H-89
blocked the cAMP activation of PI3K-C2α, and thus demonstrated a probable
involvement of PKA. In studies that have used H-89 inhibitor at 10μM (Davies, Reddy
et al., 2000) results have shown that H-89 can inhibit around another eight protein kinases
other than PKA, suggesting that the inhibitor is not very specific. However, in these
experiment we used the inhibitor at a much lower range (0.1 μM) to ensure specificity.
Such data provides the first piece of evidence that PKA is involved in the activation of
PI3K-C2α and could be of importance in future studies and also highlights that class IA
and class-II PI3K are clearly regulated by different mechanisms. Furthermore, although
the cAMP/PKA-mediated signalling pathways are amongst the most intensely studied
signalling pathways, our findings showing that PI3K-C2α can act as an effector for this
pathway, and these data will open up new avenues in this field.
Figure 6.1 Effects of adrenaline, insulin or both on class 1A p85/PI3K adapter subunit activation in soleus muscle.

Muscle extracts (soleus) were stimulated with 10mU/ml insulin and 10μM adrenaline for 30 minutes and extracts were then homogenised. Lysate containing 500μg of protein was immunoprecipitated using p85 α-specific antisera. PI3K assays were performed on these immunoprecipitates. Results are the means of three experiments carried out in duplicate and are expressed as % of basal (+/- S.E.M). Control samples were normalised and p-values were calculated using the student t-test. Where there are no p-values data did not reach statistical significance.
Figure 6.2 Effects of adrenaline, insulin or both on PI3K-C2α activation in soleus muscle.

Muscle extracts (soleus) were stimulated with 10mU/ml insulin and 10μM adrenaline for 30 minutes and extracts were then homogenised. Lysate containing 500μg of protein was immunoprecipitated using PI3K-C2α-specific antisera. PI3K assays were performed on these immunoprecipitates. Results are the means of three experiments carried out in duplicate and are expressed as % of basal (+/- S.E.M). Control samples were normalised and p-values were calculated using the student t-test. Where there are no p-values data did not reach statistical significance.
Figure 6.3 Effects of the cAMP analogue (8'-CPT'·cAMP) on PI3K-C2α activity in CHO-IR cells

CHO-IR cells were serum-starved overnight and then pre-incubated with the PKA inhibitor H-89 0.1μM for 20 minutes followed by stimulation with 100μM of the cAMP analogue 8’-CPT’·cAMP (denoted CPT) for 15mins. Cells were lysed, and lysates were subjected to immunoprecipitation using PI3K-C2α specific antisera. PI3K assays were performed on these immunoprecipitates. Results are the means of three experiments carried out in duplicate and are expressed as % of basal (+/ - S.E.M). Control samples were normalised, and p-values were calculated using the student t-test. Where there are no p-values data did not reach statistical significance.
Chapter 6 Results

**Akt Thr 308 protein levels**

<table>
<thead>
<tr>
<th>Treatment of muscle</th>
<th>Control</th>
<th>Adrenaline</th>
<th>Insulin</th>
<th>Insulin + Adrenaline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative Protein levels (%) of control</td>
<td>![Graph showing relative protein levels]</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Insulin**
- - - + +

**Adrenaline**
- + - + +

**AKT Thr 308**
Figure 6.4 Effects of adrenaline, insulin or both on the phosphorylation of PKB in soleus muscle.

Muscle extracts (soleus) were stimulated with 10mU/ml insulin and 10μM adrenaline for 30 minutes and extracts were then homogenised. 50μg of total protein was subjected to SDS-PAGE and Western blotted with antisera specific for threonine 308 AKT. Similar results were obtained at least three times.
Chapter 6 Results

% of basal

Stimulation time (minutes)

Insulin

Adrenaline

p85
Figure 6.5 Effects of adrenaline, insulin or both on the recruitment of class IA p85/PI3K adapter subunit to phosphotyrosine complexes in soleus muscle.

Muscle extracts (soleus) were stimulated with 10mU/ml insulin and 10μM adrenaline for indicated time-points and extracts were then homogenised. Lysates containing 500μg of protein was immunoprecipitated using PY99 anti-phosphotyrosine antibody. Immunoprecipitates were separated by SDS-PAGE and Western blotted with PI3K-C2α. Blot is a representative of one experiment carried out from muscle samples stimulated for 5 minutes. Similar results were obtained at least three times.
Figure 6.6 Effects of adrenaline, insulin or both on the recruitment of PI3K-C2α to phosphotyrosine complexes in soleus muscle.

Muscle extracts (soleus) were stimulated with 10mU/ml insulin and 10μM adrenaline for 30 minutes and extracts were then homogenised. Lysates containing 500μg of protein was immunoprecipitated using PY99 anti-phosphotyrosine antibody. Immunoprecipitates were separated by SDS-PAGE and Western blotted with PI3K-C2α. Similar results were obtained at least three times.
The PI3K-dependent signalling pathways have been implicated in the regulation of a number of cellular processes including: regulation of metabolism, differentiation, cell growth, apoptosis, vesicle trafficking, and cytoskeletal rearrangements (Shepherd, Withers et al., 1998; Toker and Cantley 1997). A number of PI3Ks have been identified which all share high homology in the catalytic domain. These have been sub classified, class I PI3K (which is further sub classed to class IA and class IB PI3K), class II, class III, and class IV PI3K based on shared structural and functional characteristics (Domin, Pages et al., 1997). In this thesis we have concentrated on identifying candidate pathways and mechanisms involved in the regulation of PI3K-C2α.

A wide range of stimuli have been shown to activate the class II PI3Ks, these include: activation by growth factors (Brown, Domin et al., 1999; Urso, Brown et al., 1999), integrins (Zhang, Banfic et al., 1998; Paulhe, Perret et al., 2002) and chemokines (Turner, Domin et al., 1998). Both PI3K-C2α and PI3K-C2β have been shown to be downstream signalling targets of activated EGF, PDGF receptors (Arcaro, Zvelebil et al., 2000) and insulin receptors (Brown, Domin et al., 1999; Urso, Brown et al., 1999; Brown and Shepherd 2001). The functional role of PI3K-C2α is still controversial. Evidence has been presented claiming that the class II PI3K is involved in clathrin coated vesicle trafficking (Gaidarov, Smith et al., 2001; Prior and Clague 1999), activation of PKB (Arcaro, Khanzada et al., 2002) and nuclear mRNA processing (Didichenko and Thelen 2001). Recent reports also show a biological function for class II PI3K by expressing the protein during Drosophila Melanogaster development (MacDougall, Gagou et al., 2004). These studies show that a class II polyproline motif in the catalytic subunit found in
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*Drosophila* and *C. elegans* (Vanhaesebroeck and Waterfield 1999) (PI3K_68D) can bind the Drk adaptor protein *in vitro* via the N-terminal SH3 domain of Drk. Drk is the *Drosophila* homologue of the SH2/SH3 domain adaptor protein Grb2 (Olivier, Raabe et al., 1993). This suggests a role for Drk in the localisation of PI3K_68D, thus allowing it to modify signaling pathways downstream of cell surface receptors (MacDougall, Gagou et al., 2004).

Previously it has been shown that class II PI3Ks are phospho proteins (Didichenko, Fragoso et al., 2003; Didichenko and Thelen 2001; Brown, Domin et al., 1999; Molz, Chen et al., 1996). Both PI3K-C2α (Didichenko and Thelen 2001; Brown, Domin et al., 1999) and PI3K-C2β (Crljen, Volinia et al., 2002; Brown and Shepherd 2001) have been shown to undergo a shift in apparent molecular weight in response to certain stimuli. However, it is not yet clear whether these modifications are required for activation. In the case of PI3K-C2α the bandshift appears to be caused by phosphorylation (Didichenko and Thelen 2001; Brown, Domin et al., 1999), while in the case of PI3K-C2β evidence shows that activation involves a calpain dependent proteolysis step (Crljen, Volinia et al., 2002).

Overall, the mechanisms involved in the regulation of PI3K-C2α are still not clearly understood. The present study sought to bridge this gap, and shed light into the role of PI3K-C2α activity. The first and second part of this study focused on the effects of two kinases, ERK and PKC, on the activation and phosphorylation of PI3K-C2α, and on the ability of PI3K-C2α to associate with tyrosine phosphorylated proteins. We chose to investigate the effects of insulin and PMA as both are known to activate ERK and PKC. Additionally many endpoint responses stimulated by growth factors such as insulin
overlap to some extent with phorbol esters such as PMA, for example: gene expression (Buchner 1995), membrane trafficking (Tanaka and Nishizuka 1994; Gibbs, Calderhead et al., 1991), and membrane ruffling (Kotani, Yonezawa et al., 1994). Furthermore, there were previous data from our laboratory showing that insulin induced the activation, phosphorylation of PI3K-C2α (Brown, Domin et al., 1999) and its association with a tyrosine phosphorylated protein of 160kDa; however, the mechanism by which this regulation occurred was not known. Comparing the effects of PMA and insulin using a number of strategies including inhibitors of PKC, ERK and PI3K, have highlighted that insulin and PMA use different mechanisms for the regulation of PI3K-C2α. The finding that growth factors such as insulin and phorbol esters such as PMA regulate class II PI3K immediately raises the question of how this occurs. Our results show that the ERK cascade is involved in the PMA-induced activation and phosphorylation of PI3K-C2α; whereas, PKC kinase activity is not directly involved in the activation of PI3K-C2α, but is involved in the phosphorylation of PI3K-C2α. These results firstly suggest that the effects observed on activity could be due to the newly identified phorbol esters receptors including mammalian α and β chimaerins, Ras-GRP, and Unc-13 Caenorhabditis elegans (Kazanietz 2002; Kazanietz 2000; Ron and Kazanietz 1999). Secondly, we can conclude from our results that the activation and phosphorylation of PI3K-C2α are two independent events and these data are consistent with recent reports (Didichenko, Fragoso et al., 2003). Our results also show that other classes of PI3K such as class IA or class III PI3K are involved in the insulin-induced activation and phosphorylation of PI3K-C2α.
Proteins involved in signal transduction usually have specific domains within their structure which mediate certain signalling cascades. For example, proteins that have FYVE domains, PX domains and PH domains selectively bind PtdIns-3-P (Dowler, Currie et al., 2000). Our results show a new and exciting finding indicating that phorbol esters induce the production of PtdIns-3-P as well as the activation of PI3K-C2α. The most important role of PtdIns-3-P appears to be in regulating vesicular trafficking. Evidence pointing towards the involvement of PI3K-C2α in making PtdIns-3-P include the following: (1) recent published work from our collaborators show that the insulin dependent pool of PtdIns-3-P in L6 cells is resistant to wortmannin indicating that PI3K-C2α, which is also wortmannin-resistant, is responsible for producing PtdIns-3-P (Maffucci, Brancaccio et al., 2003); (2) PI3K-C2α has a PX domain at the C-terminus. PX domains have been shown to bind PtdIns-3-P, and PX domain containing proteins have been shown to be involved in vesicular trafficking. Furthermore, PI3K-C2α has recently been shown to be have a clathrin binding domain, and subsequently to be involved in clathrin coated vesicle trafficking (Gaidarov, Smith et al., 2001; Prior and Clague 1999).

It is well established that PtdIns-3-P is localised to endosomes. However, until recent reports (Maffucci, Brancaccio et al., 2003) it was not known whether growth factors could stimulate the production of PtdIns-3-P at the plasma membrane. Therefore our findings together with our collaborators, that show that insulin and PMA stimulate the translocation of PtdIns-3-P to the plasma membrane, is a new and exciting area for the future.
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The fact that insulin and PMA use different pathways to regulate PI3K-C2α is further supported by the finding that while insulin stimulated association with the 160 kDa tyrosine phosphorylated protein as we have previously described (Brown, Domin et al., 1999), and a 106kDa phosphotyrosine protein PMA did not. Conversely PMA, but not insulin, increased association with a 120kDa tyrosine phosphorylated protein. We show that this PMA-induced phosphorylation band of 120kDa contains a member of the FAK and p130cas family of focal adhesion proteins. This finding proposes a novel role for PI3K-C2α in cellular adhesion processes.

Since we are proposing that insulin and PMA regulate PI3K-C2α by distinct mechanisms, future work could involve the mapping of phosphorylation sites by point mutational analysis. The role of these sites in PI3K-C2α regulation and localisation could be investigated. Other studies could involve the designing and making of phospho-specific antibodies designed to the potential phosphorylation sites, for example, Erk1 kinase, p38 MAPK, GSK 3 kinase, PKC, EGFR Kinase, Shc PTB and Cortactin found in the protein sequence of human PI3K-C2α using Scansite (scansite.mit.edu). These antibodies can then be used to identify which sites are phosphorylated in response to various stimuli. Mutation of these potential phosphorylation sites could also be conducted to identify which sites are required for the regulation/localisation of PI3K-C2α. Other future studies could involve the investigation of the role that the protein cortactin, a 80-85 kDa protein capable of binding to F-actin and colocalizing with actin, has on the regulation of PI3K-C2α. The role this protein plays in the cell would be of particular interest as Scansite (scansite.mit.edu) reveals a cortactin phosphorylation site in the human sequence of PI3K-C2α. Further studies could include the identification of the
insulin-induced tyrosine-phosphorylated proteins using 1-2D gels to isolate the phospho-proteins, and then use mass spectroscopy to identify the isolated proteins. The identification of the proteins could then be used to investigate its role in insulin-stimulated PI3K-C2α activation. To further substantiate the observations made with the pharmacological MEK and PKC inhibitors, future studies could investigate the effects of dominant negative mutants of these enzymes, and then assess their impact on PI3K-C2α activation.

The third part of the study focused on the effects of cytokines such as leptin and TNFα on the activation, and phosphorylation of PI3K-C2α and on the ability of PI3K-C2α to associate with tyrosine phosphorylated proteins. A wide range of stimuli have been found to activate class II PI3K including activation by integrins (Zhang, Banfic et al., 1998) and G-protein coupled receptors such as MCP-1 (Turner, Domin et al., 1998); however, there is no evidence for the involvement of cytokines. The finding that two different cytokines leptin and TNFα, which act through different receptors, both induce the activation of PI3K-C2α via ERK, is similar to the effects we observe with phorbol esters and is the first evidence for the involvement of PI3K-C2α in cytokine signalling. A review of the literature reveals that activation of ERK is not required for insulin stimulation of GLUT4 translocation in muscle (Berti and Gammeltoft 1999; Chen, Zheng et al., 1995), but it is required for the leptin and AICAR-stimulated translocation of GLUT4 translocation in muscle (Berti and Gammeltoft 1999; Chen, Zheng et al., 1995). Recently it has been found that a wortmannin-resistant PI3K most likely PI3K-C2α is involved in GLUT4 translocation (Maffucci, Brancaccio et al., 2003), so these results suggest that leptin stimulation of GLUT4 translocation requires PI3K-C2α. Although we
show that leptin induces the activation of PI3K-C2α, it is not known whether AICAR is also capable of activating PI3K-C2α, therefore, future studies could involve investigating whether AICAR can activate PI3K-C2α. To further substantiate the observations made with the pharmacological MEK inhibitors, future studies could investigate the effects of dominant negative mutants of MEK, and then assess their impact on PI3K-C2α activation.

The fourth part of the study focused on the effects of insulin and adrenaline on the activation and recruitment of class Iα and PI3K-C2α in rat soleus muscle. One of our collaborators, Dr Jorgen Jensen, had recently observed that adrenaline potentiates insulin-induced activation of PKB. Adrenaline acts via the β-adrenergic receptor to increase cellular levels of cAMP, and this pathway is particularly important in muscle which expresses high levels of the β-adrenergic receptors. Current evidence indicates that PI3K activity is required for PKB activation, with both the class I and class II PI3Ks having been implicated (Arcaro, Khanzada et al., 2002). We show that adrenaline is even more efficient than insulin at inducing the activation of PI3K-C2α (Soos, Jensen et al., 2001). Furthermore, we demonstrate that class Iα and class II are both recruited to phosphotyrosine complexes in soleus muscle upon insulin treatment. The recruitment of class Iα PI3K to tyrosine phosphorylated proteins such as IRS-1 is generally accepted as the major mechanism by which it is activated. It is therefore of interest that adrenaline modulates insulin’s effect on recruitment of PI3K to tyrosine phosphorylated proteins in a biphasic manner, with a potentiation of insulin’s effects observed at early timepoints (<10min), but an attenuation of insulin’s effects seen at later time points. The complex interaction between insulin and adrenaline is very interesting and has not previously been
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observed. We further provide the first evidence that PKA is involved in the activation of PI3K-C2α. This could be of importance in future studies, and also highlights that class IA and PI3K-C2α are clearly regulated by different mechanisms. Furthermore, the cAMP/PKA-mediated signalling pathways are amongst the most intensely studied signalling pathways. Our findings showing that PI3K-C2α can act as an effector for this pathways will open up new avenues in this field.

Going forward, one way to try and understand how PI3K-C2α functions in the cell is to use methods that downregulate PI3K-C2α expression. Such methods include creating a PI3K-C2α knockout mouse where regulation of PI3K-C2α can be investigated. However, such a technique would require a lot of time and effort but also would be very expensive to do, therefore a less expensive and less time consuming technique is preferred. Techniques that can be used for such studies, include, antisense technology (Schlingensiepen, Hausen et al., 1997) and RNAi (Elbashir, Harborth et al., 2001; Hammond, Caudy et al., 2001; Sharp 2001). The potential applications of RNAi include, (i) the study of molecular and cellular processes, (ii) target validation studies for example replacing antisense technology and knock out mice which is more time and effort consuming, and (iii) gene therapy. During our studies we obtained preliminary evidence that antisense and RNAi technologies can be used to down regulate protein expression of PI3K-C2α. Partial reductions in PI3K-C2α protein levels were obtained but lack of time prevented full optimisations of the system (data not shown).

In conclusion data presented in this thesis shows that growth factors, such as insulin, tumour promoting agents, such as phorbol esters, cytokines such as leptin and TNFα, and hormones such as adrenaline, are all involved in either the activation and/or
phosphorylation of PI3K-C2α. We conclude that multiple pathways are involved in the regulation of PI3K-C2α. For example, the PMA-induced activation of PI3K-C2α is not directly regulated by PKC activity, however, PI3K-C2α phosphorylation is, thus indicating that activity and phosphorylation are two separate events. Furthermore, we conclude that the PMA; leptin and TNFα-induced activation and phosphorylation of PI3K-C2α is dependent on ERK (See Fig 7.1). Such data represent a crucial and novel role of the MEK/ERK cascade in the regulation of PI3K-C2α. Furthermore, we can also conclude that PMA and insulin both induce the production of PtdIns-3-P at the plasma membrane. Preliminary evidence indicates that PI3K-C2α is responsible for the production of this lipid. What is more striking is the fact that the PMA-induced production of PtdIns-3-P is dependent on ERK which parallels the activation of PI3K-C2α. Furthermore, we conclude that the insulin-induced activation and phosphorylation of PI3K-C2α requires class I or class III PI3K (See Fig 7.1). Finally we show that adrenaline, as well as insulin, is involved in the regulation of PI3K-C2α in muscle (See Fig 7.1). This could be of great importance in future studies and can contribute to the understanding of the functional role that PI3K-C2α plays in the cell. Overall, work presented here in this thesis is the beginning of a very interesting and exciting chapter to the regulation and control of PI3Ks for both research and therapeutic roles for the future.
Figure 7.1 Pathways Involved in the Function of PI3K-C2α

(A) Pathways involved in the activation of PI3K-C2α

- Insulin
- PMA
- TNFα and leptin
- Adrenaline
- CAMP analogues

(B) Pathways involved in the phosphorylation of PI3K-C2α

- Insulin
- PMA
- PKC
- ERK

Increased Activity

Phosphorylation of PI3K-C2α
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