MULTIPLE MECHANISMS OF REGULATING GLUCOSE TRANSPORTERS AND

GLUCOSE TRANSPORT IN SKELETAL MUSCLE CELLS

by

Zayna A. Khayat

A Ph.D. thesis submitted in conformity with the requirements

for the degree of Doctor of Philosophy

Graduate Department of Biochemistry

University of Toronto

© Copyright by Zayna A. Khayat 2001
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.
The hidden well-spring of your soul must needs rise and run murmuring to the sea; 
And the treasure of your infinite depths would be revealed to your eyes. 
But let there be no scales to weigh your unknown treasure; 
And seek not the depths of your knowledge with staff or sounding line. 
For self is a sea boundless and measureless.

Kahlil Gibran, The Prophet

This thesis is dedicated to my parents, 

Michel and Nadia Khayat.
MULTIPLE MECHANISMS OF REGULATING GLUCOSE TRANSPORTERS AND GLUCOSE TRANSPORT IN SKELETAL MUSCLE CELLS

A Ph.D. thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Biochemistry, University of Toronto 2001

ABSTRACT
Glucose is a universal energy substrate of mammalian cells. The Thesis examines the two main pathways of regulating glucose transport in muscle cells - insulin and the metabolic demand pathway. Insulin and the mitochondrial uncoupler dinitrophenol (DNP) were used to stimulate these two pathways in L6 muscle cells in order to dissect differences involving 3 major cellular regulatory pathways: 1. the actin cytoskeleton and temporal-spatial regulation of signals and glucose transporters: 2. signalling pathways activated by these stimuli: and 3. cellular mechanisms controlling chronic glucose transporter expression. The results demonstrate that insulin provokes a rapid and marked aggregation of filamentous actin into structures that provide the coordinates for the insulin-derived signals to meet glucose transporter organelles, and direct their insertion into membrane ruffles. The results also reveal that, in contrast to insulin, DNP-stimulated glucose uptake largely depends on cytosolic Ca\(^{2+}\) and Ca\(^{2+}\)-sensitive PKCs, but likely does not engage the fuel sensing enzyme AMPK. Finally, insulin and DNP were shown to elicit different long-term effects on glucose transporter expression: whereas prolonged exposure to insulin increases glucose transporter biosynthesis, chronic exposure to DNP increases the half-life of the glucose transporters in an isoform-specific manner. By uncovering the different cellular factors accessed by insulin and DNP to control glucose transport, the Thesis enhances our understanding of the diverse means employed by the muscle cell to control glucose homeostasis, and may have implications for the therapeutic treatment of diseases associated with impaired glucose utilization, such as Type 2 diabetes.
PREFACE

The work presented in this dissertation was performed in the period of September 1996 – July 2001 in the laboratory of Dr. Amira Klip in the Programme in Cell Biology of the Research Institute of the Hospital for Sick Children in Toronto, Ontario, Canada under the academic direction of the Department of Biochemistry of the University of Toronto, Canada. Financial support to Zayna A. Khayat was provided by graduate fellowships from the Natural Sciences and Engineering Council of Canada (1996-1998), and the Canadian Institutes of Health Research Doctoral Award (1998-2001), and scholarships from the University of Toronto (Open Scholarship, 1996), Walter Sumner Memorial Foundation (1998) and Ara Mooradian Memorial Foundation (1999). Grant support to Dr. Amira Klip for the studies described in this Thesis was received from the CIHR (MA-7307), the Canadian Diabetes Association, and the Eli Lily/Banting and Best Diabetes Centre.

The work presented in this Thesis has appeared in the following papers and reviews:

Papers:


Manuscripts in Preparation:


2. Theodoros Tsakiridis, Evangelina Tsiani, Poli Lekas, Zayna A. Khayat, Gary Sweeney, Romel Somwar, Vera Cherepanov, Ba Tu, Amira Klip, Catherine Whiteside, and Gregory P. Downey. *Independent activation of phosphatidylinositol 3-kinase and Ras and their downstream effectors by insulin in L6 muscle cells.*

Reviews:


ACKNOWLEDGMENTS

Throughout my training, Dr. Amira Klip has assumed more roles than are imaginable - my mentor, my supervisor, my colleague, and my friend. Thank you Amira for setting your expectations of me so high, and for pushing me to consistently meet or exceed those expectations.

I could not possibly have asked for a better graduate committee than Drs. Klip, Reinhart Reithmeier and Margaret Rand who were instrumental to the progress of my Ph.D. project, and my evolution as a scientist. I thank them for their constant enthusiasm and support of my academic program and my decision to pursue a new career path.

The biochemistry department of the University of Toronto has been my academic liaison to the University. I will always be thankful to Dr. Charlie Deber and his then graduate secretary, Anna Vanek for enthusiastically recruiting me to this great University. It has been a pleasure to be mentored by a first-rate team of scientific thinkers and leaders comprising the Biochemistry faculty. In particular, I thank Drs. Peter Lewis and Jacqueline Segall for their tremendous leadership as chair and graduate co-ordinator, respectively. And, my friend and mentor, Patricia Bronskill for continuously guiding me to become a better teacher in higher education.

My journey to completing this dissertation was significantly enriched by the amazing team of students and fellows in the Klip lab who are too many to mention. In particular, I have formed lasting friendships with Dr. Celia Taha, Karen Yaworsky, Romel Somwar, Dr. Gary Sweeney, and Varinder Randhawa, who I also deeply thank for their many scientific insights and collaborations through the years. I am most grateful to Zhi Liu, Rami Garg, and the late Toolsie Ramial, for their sound technical and scientific assistance. And Crina Lador and Nish Patel - whom I supervised as undergraduates — I thank them for their eagerness to learn. I will forever appreciate the leadership, creativity, and personal impact shown by our research associate, Dr. Philip Bilan. Finally, I thank Dr. Leonard Foster who s unparalleled abilities to think insightfully, formulate hypothesis, and be a friend like no other are all qualities which I shall always try to emulate in my own career and personal life.

I would be remiss if I did not mention the many colleagues I have had the pleasure of working with in the Cell Biology Programme at Sick Kids. I deeply thank Sheryl Mann and Henry Knight for their excellent administrative assistance, and for their friendship. And, of course, my fellow grad students, Crestina, Mark, Hao, Cameron, Roberto, and Mahmood - thanks for your camaraderie and scholarship over the years. I am additionally grateful to our chair, Dr. Sergio Grinstein for the honor of allowing me to learn from one of Canada’s greatest scientific minds.

At last, the ‘non-scientists’ who fostered my personal development: I deeply thank my many friends and family for championing me through this long degree. I will always remember how Grase and Gary, Rob and Jeanne, and Chris and the Howard family helped me balance my arduous academic life with a social life that was equally as intense! I am forever grateful to my friend Pete Lawlor for saying ‘yes’ before I could finish asking for the favor. Finally, my thanks to the runners (Cyndie, Steph, Elizabeth), basketball players (Jennifer) and the ultimate frisbee players (Jeremy, John, Ai-fi) for making me play just as hard as I work.

My family was instrumental to me earning my Ph.D. degree. To begin, I thank Uncles Elias and Joe, and Aunts Chris and Toni for their endless support - both scholastic and financial. To my sisters and brother, Roula, Said and Reama, who I have always thought are more talented than I could ever be: thank you for never once doubting that I could achieve anything imaginable. And, to mom and dad, thank you for always being so interested in what I do, no matter how absurd it sounds! It was the work ethic and honesty which you taught me that got me through this degree.

Finally, I will forever be grateful to Sheldon Bell, my best friend and soulmate, for being there during the most difficult stage of my doctorate training - the final stretch.
# TABLE OF CONTENTS

ABSTRACT ........................................................................................................ iii
PREFACE ........................................................................................................ iv
ACKNOWLEDGMENTS .................................................................................. vi
TABLE OF CONTENTS .................................................................................. vii
LIST OF TABLES ............................................................................................ xi
LIST OF FIGURES ........................................................................................... xii
LIST OF ABBREVIATION ................................................................................ xiv

CHAPTER 1: BACKGROUND, RATIONALE AND HYPOTHESIS .................... 1

GLUCOSE HOMEOSTASIS ............................................................................... 2

GLUCOSE TRANSPORTERS OF MAMMALIAN CELLS ............................... 2
GLUT4 - Kinetic Properties and Intracellular Sorting .................................. 5
GLUT1 - Kinetic Properties and Intracellular Sorting .................................. 6
GLUT3 ............................................................................................................. 7

ACUTE GLUCOSE TRANSPORT REGULATION - TWO MAJOR PATHWAYS EXIST 8
Evidence for an Insulin-Independent Glucose Transport Pathway ................. 9
1. Additivity ..................................................................................................... 10
2. Different Intracellular GLUT4 Storage Pools ........................................ 10
3. Different Signalling Intermediaries ......................................................... 11
4. Different Membrane Traffic Intermediaries ............................................ 12
5. Resistance of GLUT4 Traffic to Insulin, but not Exercise During Insulin Resistance .......................................................... 12
Glucose Transport Regulation by Metabolic Demand ................................... 13
Physiological Activators of the Metabolic Demand Pathway ......................... 13
In Vitro Activators of the Metabolic Demand Pathway ................................ 14
Mimicking Exercise ....................................................................................... 14
Chemical Hypoxia ......................................................................................... 15
Role of Ca++ in the Metabolic Demand Pathway ........................................ 16
Role of PKC in the Metabolic Demand Pathway .......................................... 18
PKC Isoforms and their Regulation ............................................................ 18
Pharmacological Approaches to Assess the Actions of PKC Isotypes .......... 19
Studies Implicating ePKC Involvement in the Alternative Pathway ............ 20
Role of AMPK in the Metabolic Demand Pathway ...................................... 21
Other Proposed Mediators of the Metabolic Demand Pathway .................... 23
Nitric Oxide .................................................................................................. 23
Adenosine ..................................................................................................... 24
Bradykinin and G, Coupled Receptors ...................................................... 24
Ca++-Calmodulin Dependent Kinase II ......................................................... 25
Insulin-Dependent Glucose Transport Regulation ....................................... 26
The Insulin Receptor (IR) ............................................................................ 28
Insulin Receptor Substrate (IRS) ................................................................. 29
Phosphatidylinositol 3-Kinase (PI3-K) ......................................................... 32
The PI3-K Family of Enzymes ..................................................................... 33
Discerning the Role of PI3-K in Insulin Action ........................................... 35
Signals Acting Downstream of PI3-K: Akt/Protein Kinase B (PKB) and Atypical PKC (aPKC) ......................................................................................... 36
Akt/Protein Kinase B (PKB) .......................................................................... 37
Akt Family Members and their Mechanism of Activation ............................ 37
Role of Akt in Insulin Action ........................................................................ 39
aPKC (PKC), and PKCQ ............................................................................. 40
aPKC Isoforms: and their Mechanism of Activation ................................... 40
Role of aPKC in Insulin Action ............................................................... 41
Other Insulin Signalling Pathways Implicated in GLUT4 Traffic .................. 42
General Receptor for Phosphoinositides-1 (GRP1), ARF6 and Phospholipase D (PLD) 42
PI3-K-Independent Mediators of Insulin-Stimulated GLUT4 Translocation ....... 43
Heterotrimeric GTP-Binding Proteins ................................................... 43
CAP/Cbl/Flotillin/TC10 Signalling Pathway .............................................. 44

REGULATION OF GLUT4 VESICLE DOCKING AND FUSION – THE SNARE
HYPOTHESIS ......................................................................................... 45

PARTICIPATION OF THE CYTOSKELETON IN INSULIN-DEPENDENT GLUCOSE
TRANSPORTER TRANSLLOCATION ....................................................... 48
The Actin Network .................................................................................. 49
Actin Structure and Morphology ............................................................ 49
Role of Rho family GTPases in Cortical Actin Cytoskeleton Remodelling ...... 51
Biological Tools to Study Actin Function ................................................ 52
Participation of the Actin Cytoskeleton in Insulin-Regulated GLUT4 Traffic .. 54
The Microtubule Network ....................................................................... 55
Involvement of Microtubules in Insulin Action ......................................... 56
The Intermediate Filament Network ........................................................ 57
Participation of Intermediate Filaments in Insulin Action ........................... 58

SIGNALLING PATHWAY REGULATING THE ACTIVATION OF GLUCOSE
TRANSPORTERS BY INSULIN AND ENERGY DEMAND ................................ 58

PATHOLOGICAL CONDITIONS ASSOCIATED WITH IMPAIRED GLUCOSE
UTILIZATION ......................................................................................... 60

RATIONALE, HYPOTHESIS AND ORGANIZATION OF THE STUDY ................... 62

CHAPTER 3: ROLE OF THE ACTIN NETWORK IN INSULIN ACTION .................. 63

CHAPTER 4: MEDIATORS OF ACUTE GLUCOSE TRANSPORT REGULATION IN
RESPONSE TO METABOLIC DEMAND .................................................... 63

CHAPTER 5: MECHANISMS OF CHRONIC GLUCOSE TRANSPORT REGULATION IN
RESPONSE TO METABOLIC DEMAND .................................................... 64

CHAPTER 2: MATERIALS AND METHODS .................................................. 66

THE L6 SKELETAL MUSCLE CELL LINE AS A MODEL OF SKELETAL MUSCLE .... 67

REAGENTS ............................................................................................. 70

ANTIBODIES ......................................................................................... 71

CELL CULTURE AND TRANSFECTIONS ................................................... 72
Cell Lines and Cell Culture ....................................................................... 72
Construction of L6 Cells Expressing c-myc Epitope-tagged GLUT4 (GLUT4myc) .. 73
Constructs ................................................................................................ 73
Transfections ........................................................................................... 74

2-[3H]-DEOXYGLUCOSE AND 3-O-[METHYL-3H]-METHYLGLUCOSE UPTAKE ASSAY 74

DENSITOMETRIC ASSAY OF SURFACE GLUT4MYC .................................. 75

MICROSCOPY ......................................................................................... 76
Surface Scanning Electron Microscopy and Backscatter Detection ................. 76
Confocal Laser Scanning and Fluorescence Microscopy ................................. 77

MEMBRANE PREPARATIONS ................................................................... 78
Total Membrane Preparation ..................................................................... 78
Plasma Membrane-Enriched Fraction ....................................................... 79
Subcellular Fractionation ........................................................................... 80
CHAPTER 3: ROLE OF THE CYTOSKELETAL NETWORK IN INSULIN ACTION

SUMMARY

RESULTS

Insulin-Stimulated Actin Filament Remodelling
Morphological Actin Analysis
Effect of Disrupting Actin Filament Formation on Actin Remodelling and Insulin Action
Role of Rho Family G-proteins in Insulin-Dependent Actin Reorganization and GLUT4-myc Translocation to the Cell Surface
Role of the Actin Cytoskeleton in the Propagation of Insulin Signals from the Insulin Receptor to PI3-K
Role of the Actin Cytoskeleton in Propagating the Insulin Signal Transduction Pathway From PI3-K to its Effectors
Relationship Between GLUT4 Organelles and Remodelled Actin Filaments
Association of SNARE Proteins with Membrane Ruffles
Detergent Extraction of Insulin Signalling Molecules and Glucose Transporters
Biochemical Association of Insulin Signalling Molecules with Detergent-Insoluble Complexes
Biochemical Association of Glucose Transporters with the Actin Cytoskeleton

DISCUSSION

Insulin-Induced Actin Remodelling and GLUT4 Translocation
Actin as a Scaffold for Insulin Signalling Molecules
Role of the Actin Cytoskeleton in Coupling PI3-K to its Downstream Effectors
Relation of Actin Cytoskeleton Remodelling to Localization of GLUT4 Organelles and their Insertion into the Plasma Membrane
Does the GLUT4 Polypeptide Interact with the Actin Cytoskeleton?
Relationship of SNARE Proteins to Actin Structures
Impairment of Insulin Action by Perturbation of Actin Filaments

CONCLUSIONS

CHAPTER 4: MEDIATORS OF ACUTE GLUCOSE TRANSPORT REGULATION IN RESPONSE TO METABOLIC DEMAND

SUMMARY

RESULTS

DNP-Stimulated Glucose Transport is Additive to Insulin
DNP does not Engage the Insulin Signal Transduction Pathway
Role of Intracellular Ca²⁺ in DNP-Stimulated Glucose Uptake
Role of Ca²⁺-Sensitive PKC in DNP Action
GLUT4 Translocation also Depends on Ca²⁺ Mobilization and cPKC
3-O-Methylglucose Uptake also Depends on Ca²⁺ Mobilization and cPKC
Effect of Combining Ca²⁺ Chelation and cPKC Inhibition on the DNP Response
LIST OF TABLES

Table 1.1 The family of mammalian facilitative GLUT proteins ........................................ 4
Table 1.2 Classes of PKC isoforms in mammalian cells ..................................................... 18
Table 1.3 Classes of mammalian PI3-K ............................................................................. 33
Table 3.1 Relative amounts of actin and p85 in the cytosol and LDM fraction under basal conditions and in response to insulin stimulation ........................................................................ 118
Table 4.1 DNP also stimulates 3-O-methylglucose uptake in a Ca2+ and cPKC-dependent manner .................................................................................................................. 141
Table 4.2 Combination of cPKC inhibition and Ca2+ chelation does not further inhibit the DNP response .................................................................................................................. 142
Table 4.3 AICAR does not stimulate glucose uptake in L6 GLUT4myc myotubes .................. 143
Table 6.1 Summary of the major mechanisms of glucose transporter regulation by the stimuli studied in Chapter 5 ............................................................................................................. 176
LIST OF FIGURES

Figure 1.1 Model for orientation of glucose transporters (GLUT4) in the plasma membrane ........................................ 3
Figure 1.2 Structure and mechanism of action of DNP on mitochondrial respiration .................................................. 16
Figure 1.3 Chemical structures of PKC inhibitors ........................................................................................................ 20
Figure 1.4 Current model of insulin-dependent and -independent GLUT4 translocation ................................................ 27
Figure 1.5 Biological action of PI3-K ................................................................................................................................ 32
Figure 1.6 Structural features of the PI3-K inhibitor wortmannin ..................................................................................... 35
Figure 1.7 The SNARE hypothesis in GLUT4 traffic ........................................................................................................ 47
Figure 1.8 F-actin morphology of L6 skeletal muscle myotubes ......................................................................................... 50
Figure 1.9 Site of action of actin disrupting agents used in this study .............................................................................. 53
Figure 2.1 The morphology of L6 myotubes and myotubes ......................................................................................... 68
Figure 3.1 Effect of insulin on L6 myotube surface morphology ................................................................................... 94
Figure 3.2 Insulin induces dorsal, actin-rich structures which extend from the plasma membrane to the perinuclear region ................................................................. 95
Figure 3.3 Dorsal actin-rich structures cause the membrane ruffling in L6 myotubes ................................................... 95
Figure 3.4 β-actin, but not α-actin forms actin stress fibers which reorganize .......................................................... 96
Figure 3.5 Effect of actin filament disrupting compounds on basal and insulin-stimulated F-actin morphology .......... 97
Figure 3.6 Effect of actin filament disrupting compounds on basal and insulin-stimulated GLUT4/myc translocation .... 98
Figure 3.7 Dominant inhibitor Rac-1, but not Rho blocks insulin-stimulated actin filament remodelling and GLUT4/myc translocation to the cell surface ................................................. 100
Figure 3.8 PKCβ, and control IgGs do not colocalize with insulin-induced actin structures ................................ 101
Figure 3.9 The insulin receptor does not collect in insulin-induced actin structures ................................................ 101
Figure 3.10 A portion of IRS-1 colocalizes with insulin-induced actin structures ....................................................... 102
Figure 3.11 p85α and p110α, but not p110β concentrate in insulin-induced actin structures .................................. 103
Figure 3.12 PI3,4,5-P3, is generated at membrane ruffles, and overexpression of GFP-PH-GRPI inhibits insulin-stimulated actin remodelling ............................................................. 105
Figure 3.13 Overexpression of GFP-PH-GRPI prevents GLUT4/myc externalization .................................................. 106
Figure 3.14 Akt1 and phospho-Akt collect in insulin-induced actin-rich structures ................................................. 107
Figure 3.15 PKCζ and PKCα (aPKC) do not appear in insulin-induced actin-rich structures ................................... 109
Figure 3.16 Disruption of the actin cytoskeleton with CD or LB blocks aPKC activation by insulin .................... 109
Figure 3.17 GLUT4 organelles relocate to the dorsal actin-rich structures with a delay following insulin stimulation ...... 110
Figure 3.18 A portion of intracellular IRAP (a resident protein of GLUT4 vesicles) also collects in the actin structures .......... 111
Figure 3.19 The pattern of insulin-induced membrane ruffling parallels the immunostaining of GLUT4/myc on the surface of non-permeabilized myotubes ................................... 112
Figure 3.20 Immunogold-labelled cell surface GLUT4 appears in membrane ruffles ............................................ 113
Figure 3.21 Ruffle formation and appearance of increased density of immunogold-labelled cell surface GLUT4 is blocked by disrupting the actin cytoskeleton ........................................ 114
Figure 3.22 The t-SNAREs syntaxin-4 and SNAP-23 become concentrated in the insulin-induced membrane ruffles .......... 116
Figure 3.23 The v-SNARE VAMP2, but not VAMP3 colocalizes with remodelled actin .................................... 116
Figure 3.24 The content of actin and p85 increases in the LDM upon insulin stimulation ........................................ 118
Figure 3.25 Triton extraction of insulin signalling molecules ......................................................................................... 119
Figure 3.26 Triton extraction of GLUT-containing membranes .................................................................................... 120
Figure 3.27 Triton extraction of GLUT-containing membranes .................................................................................... 120
Figure 4.1 DNP-stimulated glucose uptake is additive to insulin ............................................................................. 133
Figure 4.2 DNP does not activate lipid and protein kinases activated by insulin ................................................. 134
Figure 4.3 Role of Ca2+ in the response to DNP ........................................................................................................... 135
Figure 4.4 DNP-stimulated glucose transport is reduced by the PKC inhibitor BIM-I ............................................. 136
Figure 4.5 BIM-I specifically inhibits phorbol ester-induced glucose uptake ........................................................ 136
Figure 4.6 cPKC downregulation reduces DNP-stimulated glucose transport .................................................... 137
Figure 4.7 DNP increases translocation of cPKC to plasma membrane ............................................................ 138
Figure 4.8 DNP induces a cPKC activity that is inhibitable by BIM-I ....................................................................... 139
Figure 4.9 Inhibition of PKCβ reduces DNP-stimulated glucose transport .......................................................... 140
Figure 4.10 DNP-stimulated GLUT4/myc translocation depends on Ca2+ and cPKC ........................................ 140
LIST OF ABBREVIATIONS

α-MEM, Alpha modified essential medium
AICAR, 5’-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside
AMP, Adenosine monophosphate
AMPK, Adenosine monophosphate-dependent protein kinase
aPKC, atypical protein kinase C
ARF, ADP-ribosylation factor
ATP, Adenosine triphosphate
BAPTA-AM, 1,2-bis-(2-aminophenox)ethane-V.V’.N’.N’-tetracetic acid-acetoxymethyl ester
BCA, Bicinchoninic acid
BIM, Bisindolylmaleimide
BSA, Bovine serum albumin
CaMKII, Ca2⁺ calmodulin-dependent protein kinase II
CAP, Cbl-associated protein
CB, Cytochalasin-B
CD, Cytochalasin-D
cDNA, Complementary deoxyribonucleic acid
cGMP, Cyclic guanine monophosphate
CHO, Chinese hamster ovary
CHX, Cycloheximide
Cy3, Cyanine 3
cPKC, Conventional protein kinase C
DAG, Diacylglycerol
DTT, Dithiothreitol
EGTA, Ethyleneglycol-bis-(α-aminoethyl ether)-N,N’N’,N’-tetraacetic acid
EGFP, Enhanced green fluorescent protein
ER, Endoplasmic reticulum
DNP, Dinitrophenol
F-actin, Filamentous actin
FITC, Fluorescein isothiocyanate
G-actin, Globular actin
GAP, GTPase activating protein
GER, Guanine nucleotide exchange factor
GFP, Green fluorescent protein
GLUT, Glucose transporter
GLUT4myc, Myc-tagged glucose transporter-4
GRP1, General receptor for phosphoinositides-1
GRP1-PH-GFP, GFP-tagged PH domain of GRP1
GST, Glutathione S-transferase
GTP, Guanine triphosphate
HBS, HEPES-buffered saline
HEK, Human embryonic kidney
HEPES, Hydroxyethyl piperazine-ethanesulfonic acid
IF, Intermediate filament
IR, Insulin receptor
IRAP, Insulin responsive aminopeptidase
IRS, Insulin receptor substrate
LB, Latrunculin B
LDM, Light density microsomes
MAPK, Mitogen activated protein kinase
mRNA, Messenger ribonucleic acid
MT. Microtubules
MTOC. Microtubule organizing center
NIH. National Institutes of Health
NOS. Nitric oxide synthase
NO, Nitric oxide
NP40. Nonidet P-40
NSF. N-ethylmaleimide sensitive factor
OPD. O-phenylenediamine
p38MAPK. 38 kDa mitogen activated protein kinase
p70S6K. 70 kDa S6 kinase
p85, 85 kDa regulatory subunit of PI3-K
p110. 110 kDa catalytic subunit of PI3-K
PBS. Phosphate buffered saline
PDGF. Platelet derived growth factor
PDK1/2. Phosphoinositide dependent protein kinase 1 or 2
PFA. Parafomaldehyde
PH. Pleckstrin homology
PI. Phosphatidylinositol
PI3-K. Phosphatidylinositol 3-kinase
PI3P. Phosphatidylinositol-3-monophosphate
PI-4,5-P2. Phosphatidylinositol-4,5-bisphosphate
PI-3,4,5-P3. Phosphatidylinositol-3,4,5-trisphosphate
PKB. Protein kinase B
PKC. Protein kinase C
PLD. Phospholipase D
PM. Plasma membrane
PMA. Phorbol myristic acid
PMSF. Phenylmethysulfonylfluoride
PTB. Phosphotyrosine binding
PTX. Pertussis toxin
SDS. Sodium dodecyl sulfate
SDS-PAGE. SDS-polyacrylamide gel electrophoresis
SE. Standard error
SEM. Scanning electron microscopy
SH2. Src homology 2
SN. Supernatant
SNAP. Soluble NSF attachment protein
SNAP-23/25. Synaptosome-associated protein of 23 or 25 kDa
SNARE. SNAP receptor
v-SNARE. Vesicle membrane SNARE
t-SNARE. Target membrane SNARE
SR. Sarcoplasmic reticulum
TC10. Tetracarcinoma clone 10
TCL. TC10-like protein
TLC. Thin layer chromatography
VAMP. Vesicle associated membrane protein
VAP33. VAMP associated protein of 33 kDa
ZMP. Monophosphorylated AICAR
CHAPTER 1: BACKGROUND, RATIONALE AND HYPOTHESIS

The majority of the background presented in this chapter has appeared in the following reviews:


GLUCOSE HOMEOSTASIS
The oxidation of glucose represents a major source of metabolic energy for mammalian cells. Blood glucose levels in the mammalian body are strictly maintained at approximately 5 mM by coordinated regulation between the major consumers (the brain and other tissues) and the major glucose supplier (the liver). The three main mammalian tissues where glucose derived from the diet is stored are the liver (as glycogen), skeletal muscle (as glycogen), and adipose tissue (as triglycerides). Glucose entering the circulation during a meal is rapidly taken up by these tissues, for both energy generation and storage. Of these tissues, skeletal muscle is the primary target tissue for glucose utilization after a meal (191), a regulatory mechanism controlled by the hormone insulin. Skeletal muscle is also the principal site for glucose utilization during physiological conditions of energy demand such as during exercise, where glucose is metabolized by anaerobic glycolysis and aerobic oxidation to generate ATP. Therefore the uptake of glucose by skeletal muscle during exercise or feeding is of critical importance to whole body glucose homeostasis, and is expected to be tightly regulated.

In most mammalian tissues including skeletal muscle, glucose transport across the plasma membrane is rate-limiting for its flux, and thus for glucose metabolism (234). Because the lipid bilayer of all mammalian cells is impermeable to polar molecules such as carbohydrates, the cellular entry of this important energy substrate is mediated by facilitated diffusion through specific carrier proteins located in the plasma membrane that bind glucose and transfer it across the lipid bilayer. This transport of glucose across mammalian cell membranes is mediated by a family of membrane-spanning glucose transport proteins of the GLUT family.

GLUCOSE TRANSPORTERS OF MAMMALIAN CELLS
Almost all mammalian cells express at least one subtype of the GLUT family. To date eight GLUT genes and their products have been cloned (designated GLUT1-GLUT6, and GLUT8-GLUT10). GLUT6 is a pseudogene; GLUT8, GLUT9 and GLUT10 were very
recently cloned and are not yet fully characterized (102, 321, 270); all other GLUTs encode homologous proteins which are known to transport glucose but have distinct functional properties (311). Because GLUT proteins are structurally similar and catalyze a common function, they are termed isoforms. The GLUT protein family is characterized by single polypeptides of about 500 amino acids in length. All are predicted to traverse the membrane 12 times, to expose N and C termini to the cytosol, and to be N-glycosylated between transmembrane helices 1 and 2 (Figure 1.1).

**Figure 1.1 Model for orientation of glucose transporters (GLUT4) in the plasma membrane**

Proposed structural model of the transmembrane positioning of GLUT4 based on the predicted topography. Amino acids conserved among GLUTs 1-4 are indicated by the single letter code. Other structural features include the site of glycosylation (CHO) and the possible sequences dictating intracellular sequestration (double arrows). Figure adapted from Stephens et al. (383).

Their kinetic properties are not identical, with $K_m$ values ranging from 1 to 20 mM and differing in turnover number (glucose molecules transported per unit time per transporter at saturation). All transporters display a large cytosolic middle loop between transmembrane helices 6 and 7 which is highly variable among transporters (in contrast to the more conserved transmembrane regions). Finally, the intracellular C-terminal tail of each transporter is unique.
and antibodies raised to synthetic peptides comprising 12-15 amino acids of this region are highly isoform-specific molecular probes. Glucose flux in one direction is achieved by rapid removal of free cytosolic glucose by its phosphorylation into glucose-6-phosphate by hexokinase, for its subsequent metabolism. A summary of the biological features of the family of GLUT proteins is presented in Table 1.1.

<table>
<thead>
<tr>
<th>GLUT Isoform</th>
<th>$K_m$ (mM)</th>
<th>Major Tissue Distribution</th>
<th>Function and Salient Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT1 (492 aa)</td>
<td>1-10 (26)</td>
<td>Erythrocytes, brain, blood-brain barrier, almost all cell types, cultured cells</td>
<td>Predominantly plasma membrane-associated; Constitutive glucose uptake</td>
</tr>
<tr>
<td>GLUT2 (524 aa)</td>
<td>15-20 (26)</td>
<td>Liver, kidney, pancreatic β cells, small intestine</td>
<td>Bi-directional glucose transport in liver; High capacity, low affinity; Pancreatic glucose sensor</td>
</tr>
<tr>
<td>GLUT3 (496 aa)</td>
<td>1-2 (26)</td>
<td>Neurons, placenta, platelets, fetal muscle</td>
<td>High affinity glucose transport; Primary neuronal transporter</td>
</tr>
<tr>
<td>GLUT4 (509 aa)</td>
<td>2-5 (26)</td>
<td>Skeletal and cardiac muscle, brown and white adipose tissue, brain</td>
<td>Insulin and exercise-responsive transporter; Predominantly sequestered intracellularly</td>
</tr>
<tr>
<td>GLUT5 (501 aa)</td>
<td>NT</td>
<td>Small intestine, kidney, skeletal muscle, adipose tissue, brain</td>
<td>High affinity fructose transporter; Low affinity glucose transporter</td>
</tr>
<tr>
<td>GLUT6</td>
<td>-</td>
<td>-</td>
<td>Pseudogene product (no protein)</td>
</tr>
<tr>
<td>GLUT7</td>
<td>-</td>
<td>Liver?</td>
<td>Was thought to be liver ER intracellular transporter; Cloning artifact</td>
</tr>
<tr>
<td>GLUT8/ GLUTX1 (478 aa)</td>
<td>~2 (102)</td>
<td>Testis, brain, adipose tissue, muscle, kidney, spleen, small intestine</td>
<td>Insulin responsive like GLUT4</td>
</tr>
<tr>
<td>GLUT9 (540 aa)</td>
<td>ND</td>
<td>Kidney, liver, placenta, lung, leukocytes, muscle</td>
<td>No functional studies to date; 38% identical to GLUT1</td>
</tr>
<tr>
<td>GLUT10 (541 aa)</td>
<td>ND</td>
<td>Liver, pancreas, muscle, kidney, placenta, brain, lung</td>
<td>No functional studies to date; On same locus as other diabetes genes</td>
</tr>
</tbody>
</table>

$1K_m$ for D-glucose; ND = not determined; NT = not transported; aa = amino acids

To respond to diverse demands for glucose, different tissues rely on specific properties intrinsic to the isoform(s) of GLUT they express. Moreover, the different functional roles of GLUTs are dictated by how they differ in their subcellular localization and tissue of expression. This Thesis focuses on the three GLUT isotypes known to be expressed in skeletal muscle cells: GLUT4, GLUT1 and GLUT3. The background for the Thesis will provide a detailed description of these muscle GLUT subtypes, how they are regulated by insulin and energy demand, and their dysregulation in pathological conditions of impaired glucose utilization.
GLUT4 - Kinetic Properties and Intracellular Sorting

The GLUT4 transporter isoform was first identified at the protein level with a monoclonal antibody raised to intracellular membranes of rat adipocytes (171), and its cDNA was simultaneously cloned from skeletal muscle (129), and adipocytes (38, 63, 179, 172). GLUT4 is also termed the "insulin-regulated glucose transporter" because of its characteristic regulation by this hormone (see below) and its expression in insulin-responsive tissues such as fat, and skeletal and cardiac muscles. This transporter is also expressed in fully differentiated 3T3-L1 adipocytes and L6 myotubes in culture, making these cell systems widely used to study the regulation of this transporter. The majority of reported $K_m$ values for glucose are 2-5 mM (194, 26), which are within the range of physiologic blood glucose concentrations, so GLUT4 can be half-saturated in vivo under normal glycemic conditions.

GLUT4 is distinct from all other transporters in that the protein is largely located in intracellular membranes from where it undergoes both constitutive recycling (352) and regulated exocytosis to the plasma membrane in response to an acute insulin or exercise challenge (172, 162). Specific sequences in the GLUT4 polypeptide have been identified to be required for intracellular sequestration and retrieval from the plasma membrane. These include the aromatic-based motif FQQI found at amino acids 5-8 in the N-terminus (322), and a double leucine-containing sequence at amino acids 489 and 490 in the cytoplasmic C-terminus of GLUT4 (438, 88). The morphological and biochemical characterization of the intracellular GLUT4 storage compartment(s) has been the focus of intense research and debate for the past decade. Numerous studies involving immunogold electron microscopic analysis and velocity gradient centrifugation of subcellular fractions coupled to immunoblotting have shown GLUT4 to localize to many subcellular compartments shaped as tubules, sacs, and vesicles, including the early and recycling endosomes, as well as vesicles presumed to be part of the trans-Golgi network in mature and cultured muscle and fat cells (367). The current line of thinking is that in
there may be more than one insulin-sensitive intracellular compartment endowed with GLUT4. one that contains many recycling proteins (including GLUT1, see below) and one that contains a distinct subgroup of proteins excluding GLUT1 (336). It is possible that the specialized GLUT4-containing compartment may be generated by a regulated budding process from the endosomal compartment, subsequent to internalization of GLUT4 from the plasma membrane into the endosome. GLUT4 can putatively redistribute from any of its putative donor intracellular membrane compartments to the cell surface of muscle cells (plasma membrane and transverse tubules) in response to signals elicited by stimuli such as insulin and physical exercise. The current understanding of the mechanism of redistribution of GLUT4 is discussed in further detail later.

**GLUT1—Kinetic Properties and Intracellular Sorting**

GLUT1 was the first GLUT cDNA to be cloned (285). Although it is the predominant transporter of the blood-brain barrier, GLUT1 is ubiquitously expressed in most mammalian cells where it is thought to provide basal cellular glucose uptake because of its significant localization at the plasma membrane of many cell types (286). Reports of the $K_m$ of GLUT1 for glucose range from 1 to 25 mM glucose, however the most detailed studies assign a $K_m$ to GLUT1 of about 6-7 mM depending largely on the conditions of the assay (zero-trans or equilibrium exchange) and the hexose used (D-glucose, or the non-metabolizable 3-O-methylglucose and 2-deoxyglucose) (58, 141, 26). These kinetic parameters suggest that at the post-absorptive physiological circulating levels of glucose (4-6 mM) GLUT1 is about half-saturated.

GLUT1 levels are significantly lower than those of GLUT4 in mature fat and muscle tissue. Nonetheless, the significant expression of GLUT1 in fat and skeletal muscle cells in culture has prompted a comparison of its intracellular localization to that of GLUT4. It has been
calculated that the ratio of endogenous levels of GLUT1:GLUT4 in 3T3-L1 adipocytes is around 3:1 (55), and 1:1 in L6 myotubes (462). Like GLUT4, GLUT1 also experiences basal state recycling and insulin/exercise regulated traffic in muscle cells (418) and adipocytes (492, 474) in culture. In contrast to GLUT4, GLUT1 is predominantly plasma membrane localized in the basal state and hence the fold change in GLUT1 content in response to external stimuli is less than that of GLUT4. There is debate whether GLUT1 and GLUT4 exist in distinct intracellular compartments, or whether their different steady state distributions can simply be explained by a more stringent intracellular retention of GLUT4 vs. GLUT1. Hence, it is conceivable that, while coexisting in intracellular compartments, GLUT1 continuously buds out for recycling to the plasma membrane, while GLUT4 is less apt to do so. Additionally, it is possible that the time of residence of GLUT1 at the cell surface is higher than that of GLUT4, by virtue of so far undefined retention mechanisms at the plasma membrane. A contrasting view has also been proposed, whereby both GLUT1 and GLUT4 enter the same sorting endosome upon their internalization from the cell surface, but only GLUT4 is sorted out into a more static and exclusive compartment (the true GLUT4 vesicle) which is only mobilized in response to insulin or physical exercise. Support for both models has been reported [reviewed in (91, 336)].

**GLUT3**

After the discovery of GLUT1, other transporters were identified through cDNA cloning using low stringency screening of cDNA libraries with DNA probes containing nucleotide sequences of GLUT1. In this way, GLUT3 was cloned from a fetal human muscle cDNA library in 1988 (193). GLUT3 has the highest affinity for glucose of all known members of the GLUT family, with a $K_m$ ranging from 1-2 mM. GLUT3 expression is greatest in the mammalian brain (neurons) (260, 230), placenta (13), lymphocytes (114), and platelets (89). In human muscle, GLUT3 has classically been thought to be important for fetal skeletal muscle
glucose transport since GLUT3 mRNA is abundant at this stage of muscle development (193). More recently, a role for GLUT3 in mature skeletal muscle has been proposed due to the detection of GLUT3 mRNA and protein in human muscle (386, 131). However, compared to GLUT4 and GLUT1, little is known about the regulation of GLUT3 subcellular expression and intracellular traffic in skeletal muscle. L6 muscle cells in culture, like regenerating and developing muscle, express GLUT3, making them an important model system for characterizing the role of GLUT3 in differentiated muscle glucose homeostasis. In these cells, it was previously determined that the molar ratio of surface GLUT1:GLUT3:GLUT4 is near 1.0:1.0:1.0 (462). However, their subcellular distribution differs: whereas GLUT4 is largely intracellular, GLUT3 is mostly exposed at the surface, although a small intracellular pool of GLUT3 can be translocated to the plasma membrane in response to physiological activators of glucose transport (462).

ACUTE GLUCOSE TRANSPORT REGULATION – TWO MAJOR PATHWAYS EXIST

During a meal, the rise in blood glucose levels prompts the pancreatic release of insulin which binds to its receptor expressed on most target cells. Only muscle and fat cells respond to insulin with a major increase in glucose uptake due to the selective expression of GLUT4 in those tissues. Insulin-stimulated glucose transport results from an increase in the maximal velocity of transport ($V_{\text{max}}$) without an appreciable change in the substrate concentration at which glucose transport is half maximal, or $K_m$ (294, 310). During the rapid action of insulin, there is no net change in the total cellular complement of glucose transporters; therefore the increase in transport $V_{\text{max}}$ must occur through an increase in the rate that each GLUT transports glucose (turnover number), and/or an increase in the number of functional GLUT proteins present in the plasma membrane. Indeed, the latter mechanism was substantiated as early as
1980 when a gain in glucose transporters (measured as cytochalasin B-binding sites) and glucose transport activity was seen in isolated plasma membranes from rat adipocytes (90, 393). Conversely, a decrease was detected in isolated intracellular light microsomes. This phenomenon was also observed with diaphragm (456) and hindlimb skeletal muscle membranes (213) a few years later. Soon after, it was identified that the predominant isoform of glucose transporter mobilized is GLUT4 (172) which cycles to and from the plasma membrane in the resting and insulin-stimulated states. Insulin shifts the steady state distribution of GLUT4 from its preferred intracellular residence in the basal state, to a new equilibrium where the transporters are largely retained at the cell surface of all three major tissues where glucose uptake is insulin-regulated: skeletal muscle (213, 456), cardiac muscle (483), and adipocytes (90, 393). The phenomenon is also emulated by the corresponding cells in culture (55, 462). In skeletal muscle the surface membranes include the plasma membrane itself and the transverse tubules (T-tubules) (52), which are invaginations of the cell surface that penetrate deeply into the muscle fiber. T-tubules cover about 7-times more surface area than the plasma membrane (55). By causing translocation of GLUT4 to both the plasma membrane and the T-tubules (261, 103, 454), glucose is ensured to reach the depths of the muscle fibers.

**Evidence for an Insulin-Independent Glucose Transport Pathway**

Soon after the seminal discovery of insulin-stimulated GLUT4 translocation, evidence accumulated of the existence of a second mechanism of increasing muscle glucose flux. Like insulin, the rapid uptake of glucose into a contracting muscle during a bout of exercise also results from GLUT4 redistribution to the plasma membrane and T-tubules of skeletal muscle cells (106, 162, 346). Thus, mammalian muscle cells have the capacity to adapt to situations of high metabolic demand by increasing the levels of GLUT4 at the cell surface to enhance glucose flux for anaerobic ATP production. In addition to physical exercise, these energy stressors
include hypoxia (59), environmental stress (75) and metabolic challenges to the oxidative chain (23) (all discussed in further detail below). As insulin-stimulated glucose uptake is critical to glucose homeostasis, the effectiveness of the cellular response to energy demand in terms of ATP homeostasis is critically dependent on the degree of enhancement of GLUT4 translocation and glucose uptake.

Although muscle cells respond to both insulin and energy demand by rapidly increasing the appearance of GLUT4 molecules in the plasma membrane, there is mounting evidence that these two stimuli engage independent cellular pathways to mobilize GLUT4. The notion of an "insulin-dependent" and an "insulin-independent" pathway of rapid glucose transport stimulation results from observations of numerous contrasts between insulin and metabolic stressors in their mechanisms of glucose transport activation in skeletal muscle:

1. Additivity

The best evidence that these stimuli activate glucose transport by different pathways comes from studies showing that the increase in muscle glucose transport when a maximal contraction stimulus is combined with a maximal insulin stimulus is greater than the effect of either contraction or insulin alone (323, 296). The maximal effects of insulin and hypoxia on glucose uptake into muscle are also additive (480). This additive effect of the two stimuli on glucose transport is consistent with the hypothesis that they act independently.

2. Different Intracellular GLUT4 Storage Pools

The additive effect on glucose uptake described above suggests that insulin and contractions recruit separate intracellular pools of GLUT4 to the cell surface in muscle cells. Subcellular fractionation studies using rat membranes have proven this to be the case in skeletal muscle (79, 108). In these studies, insulin, but not exercise decreases glucose transporters from a novel intracellular microsomal membrane fraction. Elegant electron microscopy analysis of GLUT4
localization in rat muscle fibers uncovered two types of intracellular GLUT4 depots, large and small ones (324). The large GLUT4 pools constitute approximately 25% of intracellular GLUT4 and are associated with the trans-Golgi network and endosomes which exclude the small GLUT4 depots. Interestingly, simultaneous stimulation with insulin and contraction results in the additive translocation of GLUT4 to both the plasma membrane and T-tubules from both depots. Similar findings were recently reported by Tomas and colleagues (412). Furthermore, colocalization of transferrin receptor (TfR) and GLUT4 is increased by insulin and decreased by contractions, suggesting that TfR-positive depots are recruited only by contractions. Nevertheless, although the insulin- and exercise-regulated membrane compartments have different sedimentation coefficients, the major protein composition of the two compartments hardly differs (79). Taken together, the above findings lead to the hypothesis that the intracellular pool of GLUT4 which is translocated in response to insulin is functionally, but perhaps not anatomically, distinct from the GLUT4 pool which is translocated in response to exercise.

3. Different Signalling Intermediaries

The past decade saw major advances in our understanding of signalling pathways engaged during GLUT4 mobilization by insulin and physical exercise, allowing for the examination of whether these two stimuli share common signalling intermediaries. It is now known that whereas insulin engages the key enzyme phosphatidylinositol 3-kinase (PI3-K, discussed in detail below) for GLUT4 translocation, PI3-K does not participate in the response to exercise (248, 139, 477). Furthermore, the increase in intracellular Ca\(^{2+}\) during muscle contraction has long been considered a critical initiator/mediator of contraction-stimulated glucose uptake (discussed in great detail below), but not insulin-stimulated glucose uptake [reviewed in (165)]. The PI3-K-dependence and Ca\(^{2+}\)-independence of insulin action (and vice
versa for exercise) lends compelling support to the view that the two stimuli act via different pathways to increase GLUT4 translocation and glucose uptake.

4. Different Membrane Traffic Intermediaries

Two proteins that have been identified as components of GLUT4-containing compartments in skeletal muscle include the vesicle fusion protein, vesicle-associated membrane protein 2 (VAMP2, described further below) (357), and insulin responsive aminopeptidase (IRAP, described further in Chapter 1), a protease with no known function (388). Both IRAP and VAMP2 have been shown to translocate along with GLUT4 in response to physical exercise in skeletal muscle (79, 231), reminiscent of the effect elicited by insulin on the distribution of these proteins. In contrast, there are differences between exercise and insulin on the redistribution of the small Ras-related GTP-binding protein Rab4. Rab proteins are molecular switches in all steps of vesicular traffic activity, and catalyze membrane traffic events through conversion from an inactive GDP-bound form to an active GTP-bound state (430). The Rab4 isoform is thought to be involved in insulin-stimulated GLUT4 translocation because it associates with GLUT4-containing compartments in the basal state (4) and redistributes to the cytosol upon insulin stimulation (87, 341). Interestingly, although insulin mobilizes Rab4 from internal membranes to the cytosol, contraction has no effect on Rab4 distribution (362). This observation supports the hypothesis that transporters stimulated to externalize in response to insulin and exercise originate from different locations in the cell, and/or respond to different traffic control and signalling mechanisms.

5. Resistance of GLUT4 Traffic to Insulin, but not Exercise During Insulin Resistance

The ability of insulin to stimulate GLUT4 translocation (and therefore glucose uptake) in muscle and fat cells is abnormally diminished in pathophysiological states associated with marked insulin resistance such as Type 2 diabetes (described in detail later). Interestingly,
whereas insulin-stimulated skeletal muscle glucose uptake and GLUT4 translocation is decreased in several animal models of insulin resistance (214, 332, 202, 363) and in Type 2 diabetic patients (180, 490, 489), the response to contraction-stimulated glucose uptake is unimpaired (46, 203, 262, 198, 338). Furthermore, the exercise-induced activation of AMPK, an enzyme implicated in the exercise pathway (see below), is intact in skeletal muscle of subjects with Type 2 diabetes (288). That skeletal muscle in Type 2 diabetes is insulin resistant, yet remains sensitive to exercise is consistent with the idea that exercise and insulin stimulate muscle glucose transport by distinct mechanisms. Indeed, many current animal models of insulin resistance have become powerful tools for elucidating the mechanisms underlying exercise- and insulin-stimulated glucose transport.

Collectively, these findings show that exercise utilizes different mechanisms from insulin to increase muscle glucose influx. Several mechanisms have recently emerged as exciting candidates that mediate this response. The mediators of the exercise pathway are discussed in the next section, and later, the insulin-derived signals are reviewed.

**Glucose Transport Regulation by Metabolic Demand**

**Physiological Activators of the Metabolic Demand Pathway**

In addition to physical exercise, glucose transport in the muscle is rapidly enhanced during other situations of compromised oxidative metabolism. Over forty years ago it was shown that incubation of skeletal muscle under hypoxic conditions leads to an increase in glucose transport as a secondary response to the inhibition of oxidative phosphorylation caused by hypoxia (334, 282). It is now known that skeletal and cardiac muscle, as well as muscle and fat cells in culture (L6 myotubes and 3T3-L1 adipocytes), manifest an enhancement of glucose transport in response to hypoxia by translocation of GLUT4 (and to some extent GLUT1) to the cell surface (23, 458, 389). From these finding it was proposed that the decrement in cell
ATP:AMP ratio (or “energy charge”) associated with inhibiting oxidative metabolism is the trigger for glucose transport stimulation. Because exercise-stimulated glucose transport is also coupled to the energy charge of the muscle cell, it would be reasonable to expect that exercise and hypoxia share common mechanisms in activating GLUT4 translocation and glucose transport in muscle, which are distinct from the insulin response. Indeed, like exercise, hypoxia-stimulated glucose uptake is also additive to insulin (334, 282), is PI3-K-independent (477) and remains intact in insulin-resistant skeletal muscle (14). Furthermore, like hypoxia and exercise, ischemic conditions are also able to stimulate glucose transport normally in insulin-resistant human skeletal muscle (299). Therefore, together with exercise, hypoxia has become a useful tool for dissecting the cellular mechanism underlying the alternative pathway of glucose transport stimulation.

**In Vitro Activators of the Metabolic Demand Pathway**

Because muscle cells in culture possess the machinery to translocate GLUT4 to the plasma membrane, they have become an important first line of experimentation to understand glucose transport stimulation in a myogenic phenotype. Muscle cells in culture are also easy to grow and manipulate experimentally compared to mature muscle tissue from rodents or humans. Moreover, muscle cell lines have been significantly useful to understand differences between insulin and other activators of glucose transport (metabolic stress). Indeed, exercise- and hypoxia-stimulated glucose uptake can be pharmacologically recapitulated in muscle cells *in vitro* as follows.

**Mimicking Exercise**

By exposing muscle cells to an increase in extracellular $K^+$, the membrane becomes depolarized and intracellularly stored $Ca^{2+}$ is released into the myoplasm (110), reminiscent of the $Ca^{2+}$ burst which occurs during the initial phases of excitation-contraction coupling. It was
recently shown that cultured H9c2 myotubes respond to an increase in extracellular K⁺ by translocating GLUT4 to the plasma membrane by a mechanism distinct from insulin (482). Therefore K⁺ depolarization of H9c2 myotubes will be a potentially useful model system for testing activators of contraction-stimulated glucose uptake in vitro.

Chemical Hypoxia
Like exercise, the stimulatory effect of hypoxia can be mimicked in cells in culture by exposure to pharmacological inhibitors (cyanide, cobalt, azide, rotenone) and uncouplers (dinitrophenol, oligomycin, CCCP) of mitochondrial respiration. This "chemical hypoxia" is associated with a transient fall in intracellular ATP levels in skeletal muscle in vivo (151) and in vitro (23). In cell systems examined, exposure to mitochondrial disruptors leads to an elevation in glucose uptake within minutes (170), reminiscent of the response to hypoxia in vivo. Thus challenges to aerobic metabolism with these chemical agents might provide vital clues to understanding the glucose transport pathway activated by metabolic demand.

For the studies presented in this thesis, the chemical employed to gain this understanding was dinitrophenol (DNP). This weak base is an uncoupler of oxidative phosphorylation which rapidly decreases cellular ATP levels in L6 muscle cells (23) (Figure 1.2). This stimulation resembles that by exercise or hypoxia rather than that by insulin since it does not require the activation of PI3-K (418). Furthermore, DNP causes translocation of GLUT4 and GLUT1 to the cell membrane, whereas insulin additionally mobilizes GLUT3 (418). Finally, unlike insulin, DNP does not require an intact actin cytoskeletal network (discussed in detail further below) to mediate its effects on glucose transporter traffic. These properties of DNP-dependent glucose uptake in cultured L6 myotubes make this a useful system to explore the regulation of the metabolic demand pathway vis a vis that of insulin.
The weak base, DNP (D'/OH in scheme) shuttles H⁺ across the inner mitochondrial membrane resulting in the dissipation of the H⁺ gradient required for ATP production.

**Role of Ca²⁺ in the Metabolic Demand Pathway**

Although the mediators of the metabolic demand pathway are largely unknown, a distinguishing feature of exercise-stimulated glucose uptake compared to the insulin-dependent pathway is the dependence of the former on changes in intracellular Ca²⁺. The spike of Ca²⁺ released from the sarcoplasmic reticulum (SR) upon depolarization of the plasma membrane in the initial phase of muscle contraction leads to myosin ATPase activation. In addition to the role of Ca²⁺ in developing tension in the muscle fiber, several studies provide indirect evidence that the rise in Ca²⁺ mediates and/or initiates contraction-stimulated glucose transport (165). Crude approaches using agents which induce a rise in cytosolic Ca²⁺, reveal an associated increase in glucose uptake rates in skeletal muscle (75, 163, 164). Conversely, preventing SR release of Ca²⁺ with dantrolene, inhibits the glucose uptake response to contraction (113, 306). Although these studies provide compelling evidence for the involvement of Ca²⁺ in the exercise
pathway, it is not likely that Ca\(^{2+}\) itself directly activates the glucose transporters because of the short duration of the Ca\(^{2+}\) burst after contraction (sub-milliseconds) compared to glucose transport which remains elevated even after a bout of exercise ends. It is proposed that the rise in intracellular calcium leads to the activation of a signalling cascade(s) that triggers the translocation of GLUT4 in the contracting muscle. The potential signalling intermediaries in the metabolic demand pathway are explored in detail below.

Given the similarities between exercise- and hypoxia-stimulated glucose uptake, an early model for how Ca\(^{2+}\) triggers hypoxia-stimulated glucose uptake was put forward (485). It is hypothesized that ATP levels negatively regulate glucose transport such that normal cellular ATP levels repress glucose transport. The transient fall in cell ATP concentration following hypoxia or inhibition of oxidative phosphorylation would slow the Na\(^+-K^+\) pump. The ensuing membrane depolarization (very small, if any) reduces Ca\(^{2+}\)-ATPase action and/or enhances Na\(^+/Ca^{2+}\) exchange or Ca\(^{2+}\)-channel opening, resulting in a rise in cytosolic Ca\(^{2+}\), which could stimulate GLUT4 translocation as occurs during physical exercise. Furthermore, low O\(_2\) levels also elicit mitochondrial Ca\(^{2+}\) release. In line with this premise are results which demonstrate that exposure to hypoxia or chemical inhibitors of oxidative phosphorylation raise intracellular Ca\(^{2+}\) levels (75).

In contrast, it is generally accepted that insulin does not change intracellular Ca\(^{2+}\) levels to anywhere near the rise caused by contraction [(211) and references within]. A more recent study revealed, however, that insulin causes local Ca\(^{2+}\) bursts in microdomains near the plasma membrane of skeletal muscle cells via activation of L-type Ca\(^{2+}\) channels (49). Therefore a small gradient of Ca\(^{2+}\) near the membrane may be required for insulin-stimulated GLUT4 translocation to the cell surface, although the ion likely triggers different cellular events than those elicited in the exercise response. In addition, there is one recent report of a requirement
for intracellular Ca\(^{2+}\) for insulin-dependent glucose uptake in 3T3-L1 adipocytes (461), suggesting that the ion may be hormone-sensitive in fat cells but not in muscle cells.

**Role of PKC in the Metabolic Demand Pathway**

**PKC Isoforms and their Regulation**

One of the best-studied targets of Ca\(^{2+}\) in cells is the serine/threonine kinase, protein kinase C (PKC). The many isoforms in the continuously growing family of PKC proteins regulate a large variety of cellular processes in response to diverse stimuli. Twelve different PKC isozymes have been identified to date and are classified into three categories based on structural characteristics and their activation by different cofactors [reviewed in (297)]. The conventional class (cPKC) include isoforms α, βI, βII, and γ and are activated by diacylglycerol (DAG) and by Ca\(^{2+}\) binding to the protein's C2 domain (302). The novel class (nPKC isoforms δ, ε, η, θ, and μ) and the atypical class (aPKC isoforms λ, ζ, and τ) are not Ca\(^{2+}\)-sensitive, but are regulated by DAG and acidic phospholipids, respectively (302, 301). Table 1.2 summarizes the current knowledge of 12 isoforms comprising the PKC sub-classes and their regulation.

<table>
<thead>
<tr>
<th>CLASS</th>
<th>ISOFORMS</th>
<th>STRUCTURE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional (cPKC)</td>
<td>α, βI, βII, γ</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Novel (nPKC)</td>
<td>δ, ε, η, θ, μ</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Atypical (aPKC)</td>
<td>λ, ζ, τ</td>
<td><img src="image" alt="Structure" /></td>
</tr>
</tbody>
</table>

Developed from (271). DAG = diacylglycerol; C1, C2, C3, C4 = domains in PKC proteins; PIPs = polyphosphoinositides.

In the absence of any stimulus, the catalytically competent but unstimulated PKC enzymes are localized in the cytosol. Activation of cPKC normally results from generation of
DAG and inositol 1,4,5-trisphosphate (IP₃) from phospholipase C-dependent phosphatidylinositol 4,5-bisphosphate breakdown. IP₃ is a potent activator of Ca²⁺ release from intracellular stores. Ca²⁺ levels may also be elevated by other stimuli which cause store release (i.e., exercise) or by the opening of plasma membrane Ca²⁺ channels. Together DAG and Ca²⁺ synergistically activate cPKC by increasing its affinity for membranes. DAG produced in membranes serves as a hydrophobic anchor to recruit the enzyme to the membrane and increase its activity and to increase the affinity of cPKC for Ca²⁺. This allows for full activation of cPKC at low micromolar concentrations of the ion. Unlike cPKC, nPKC can be recruited and activated in the membrane by DAG alone, likely due to the ability of phosphatidylinerine or other phospholipids in the membrane to activate nPKC in the absence of Ca²⁺. The third class, aPKC, can be activated by phospholipid products of activated phosphatidylinositol 3-kinase (PI3-K, discussed in detail below) generated in the membrane. The interaction of all PKC subclasses with membranes is thought to remove the pseudosubstrate portion of the enzyme from the catalytic site thereby potentiating PKC catalytic activity.

Pharmacological Approaches to Assess the Actions of PKC Isotypes

Many selective PKC inhibitors have been developed which target structural regions of PKC such as the regulatory domain or catalytic domain. One of the first identified PKC inhibitors was the microbial alkaloid, staurosporine, which competes with ATP binding (Figure 1.3). Although staurosporine potently inhibits PKC, the agent also competes with ATP for the ATP-binding sites of many other kinases and is therefore not very selective. However, staurosporine has served as a basic compound from which several novel structurally related PKC inhibitors have been developed, including the bisindolylmaleimide (BIM) PKC inhibitor used in Chapter 4. Although BIM compounds are less potent than staurosporine, they show great selectivity for different PKC isoforms. Importantly, BIM inhibits PKC activity with IC₅₀
values over 5000 times lower than that for other kinases such as that of the epidermal growth factor receptor (414). Furthermore, BIM (also known as G66850) preferentially inhibits cPKC isoforms with 10-fold greater selectivity than aPKC isoforms (263). Thus the demonstrated potency and PKC isoform specificity of BIM makes it a useful pharmacological tool for discerning the role of cPKC and aPKC. The structure of BIM is shown in Figure 1.3.

Figure 1.3 Chemical structures of PKC inhibitors

![Chemical structures of PKC inhibitors](image)

Although BIM is a potent cPKC inhibitor, the compound is not selective for specific cPKC isotypes within the conventional subclass (e.g. α, βI, βII, γ). One of the most promising isoform-selective inhibitors developed to date is the BIM derivative developed by Eli Lilly and Co. termed LY37976. Isoform-specific inhibition of PKCβ is observed through a 14-membered macrocyclic group containing a N-N'-bridged BIM moiety (175). The compound inhibits PKCβI and PKCβII in the nanomolar range and shows 70-fold greater selectivity over inhibition of PKCα (175). Since the development of LY37976, much information has been generated regarding the role of PKCβ isoforms in pathological conditions such as diabetic complications (290).

Studies Implicating cPKC Involvement in the Alternative Pathway

Because PKC proteins are implicated in several membrane traffic and membrane transport processes, they are attractive candidates for the regulation of glucose transport systems. Indeed, it is now well accepted that the aPKC proteins, PKCδ and PKCζ are involved in insulin-dependent GLUT4 translocation in L6 muscle cells (18), rat adipocytes (378), and 3T3-L1 adipocytes (17) in culture (discussed in detail later). Conventional PKC has long been
considered a candidate Ca\(^{2+}\)-sensitive effector that may participate in exercise-stimulated glucose transport in skeletal muscle (154). Indeed, skeletal muscle expresses high levels of Ca\(^{2+}\)-sensitive PKC (cPKC) [\(\alpha, \beta I, \beta II\) isoforms, (470)] and lower levels of nPKC [\(\epsilon, \eta, \delta\) isoforms, (166)] and aPKC [\(\zeta\) isoform, (470)] which do not require Ca\(^{2+}\) for activation. Although PKC activity increases in skeletal muscle during physical exercise (340), evidence that this PKC activation is required for contraction-stimulated glucose uptake is not as convincing. Pharmacological strategies interfering with PKC function have been associated with decreases in contraction-stimulated glucose transport (76, 155). However, the PKC inhibitors used at the time had very poor selectivity, and those experiments were performed before many of the PKC isoforms were identified. Therefore the involvement of cPKC in the alternative glucose transport pathway requires further investigation.

**Role of AMPK in the Metabolic Demand Pathway**

In addition to intracellular Ca\(^{2+}\), the energy status of the cell has been proposed to regulate muscle glucose uptake during the metabolic response to energy demand (449). A major cellular fuel sensor is 5'-AMP-activated protein kinase (AMPK) (464, 197), an enzyme that can be activated by various energy stressors (151). AMPK is a heterotrimeric protein with a catalytic (\(\alpha\)) subunit and two noncatalytic (\(\beta, \gamma\)) subunits (148). Of the two \(\alpha\)-subunits identified, the \(\alpha_2\) isoform is highly expressed in skeletal muscle (439), suggesting a physiological role for AMPK in this tissue. The enzyme is activated by phosphorylation during physiological conditions that deplete cellular ATP such as exercise (463) and hypoxia (235). AMPK activation largely depends on the AMP/ATP ratio through a complex mechanism that relies on allosteric regulation as well as phosphorylation by an upstream kinase, AMPK kinase (AMPKK) (382). Once activated, AMPK can inhibit anabolic reactions such as glycogen, fatty acid, and cholesterol synthesis, and promote catabolic reactions that generate ATP such as
glycolysis, and fatty acid oxidation. These observations support the role of AMPK as a potential fuel sensor of the cell.

AMPK can be pharmacologically activated by the nucleoside 5'-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) which is taken up and metabolized by tissues to form the monophosphorylated AICAR (ZMP), an AMP analog. Recently, AMPK has received considerable consideration as a potential signalling molecule involved in the metabolic demand pathway. AICAR-treated skeletal or cardiac muscle display increased glucose uptake in a manner which is additive to insulin, but not contraction, and is PI3K-independent (348, 1). Conversely, in 3T3-L1 adipocytes, AMPK activation by AICAR significantly inhibits insulin-dependent glucose uptake (350), suggesting that muscle and adipocytes require AMPK activation for different metabolic functions. Although the evidence supporting a role for AMPK in the metabolic demand pathway is compelling, it has largely been based on a correlation between AMPK activity and glucose uptake or GLUT4 translocation. The available non-specific inhibitors of AMPK, iodotubercidin and adenine 9-β-D-arabinofuranoside (araA), have been used to characterize the role of AMPK in glucose uptake. While these pharmacological agents can decrease AICAR-stimulated glucose uptake in muscle, they do not appear to inhibit glucose uptake stimulated by fuel-depleting agents such as contraction, hypoxia, and DNP (97, 287). A more conclusive understanding of the true function of AMPK in the metabolic demand pathway will be possible with the development of knockout or mutant AMPK mice. Indeed, it has now been shown that hypoxia- and AICAR-stimulated glucose uptake are completely blocked in skeletal muscle of mice expressing a dominant inhibitory mutant of AMPK, whereas the response to physical exercise is modestly reduced (284). In all, with the exception of one study (98), there is a significant correlation between AMPK activation and the stimulation of glucose uptake by energy stressors.
Other Proposed Mediators of the Metabolic Demand Pathway

The roles of Ca\(^{2+}\), cPKC and AMPK in the metabolic demand pathway are examined in Chapter 4. In addition to these three intermediaries, several novel putative mediators of the energy demand glucose transport pathway have been independently proposed in the past half-decade. Although these pathways are not explored in this Thesis, the evidence for the involvement of each of these mediators is described briefly below.

Nitric Oxide

It is well known that exercise promotes nitric oxide (NO) production in skeletal muscle. Because NO synthase (NOS) requires Ca\(^{2+}\) as a cofactor for activation (355), it has been proposed that calcium release from the SR during excitation-contraction coupling stimulates NO production. There are now data to suggest that, in addition to its role as a local modulator of blood flow, endogenously produced NO may mediate contraction-induced glucose uptake. The hypothesis of NO-dependent exercise-stimulated glucose transport is based on two main findings. The first is that giving rats a NOS inhibitor prior to exercise prevents the exercise-induced increase in plasma membrane GLUT4, but has no effect on the insulin pathway (344). These results are consistent with the hypothesis that exercise and insulin act through distinct signalling mechanisms. Secondly, exogenous NO donors increase glucose transport in rat skeletal muscle in a manner which is additive to insulin, but not additive to contraction-stimulated glucose uptake (16. 481), and is insensitive to the PI3-K inhibitor wortmannin (115). Furthermore, it was recently proposed that NO production is AMPK-dependent (126), thereby providing a link between the AMPK pathway and NO in contraction-stimulated glucose uptake.

In contrast to the above findings implicating a role for NO in the alternative pathway, several studies report discrepant findings. In isolated rat muscle and L6 myotubes, certain NO donors actually inhibit insulin-stimulated glucose uptake (187): others have shown that NO donors stimulate glucose transport additively with contraction, and not insulin (160). Furthermore.
reports show that NOS inhibition has no effect on calcium/contraction-stimulated glucose uptake in isolated rat muscles (115, 160), but inhibits the response to insulin (347). Taken together, these results indicate that NO may participate in a pathway distinct from insulin or contractions, possibly involving cGMP (115). Therefore, whether NO is truly a Ca\(^{2+}\)-dependent autocrine or paracrine mediator of glucose uptake during physical exercise remains controversial.

**Adenosine**

Another potential autocrine/paracrine regulator of muscle glucose uptake is adenosine, the production of which is markedly enhanced in the muscle or interstitial space during muscle contractions (2). A small fraction of the adenosine produced during contractions is exported from the muscle cell into the interstitial space by a combination of simple and carrier-mediated diffusion. Adenosine is thought to act as a “local hormone” by binding to adenosine receptors present in the membrane of adjacent cells, including skeletal muscle. The first suggestion that adenosine may mediate insulin-independent glucose uptake came from observations of an additivity of adenosine and insulin in glucose transport stimulation in different insulin-sensitive tissues including skeletal muscle and adipocytes (408, 244). However, there have been conflicting reports on the role of adenosine in the alternative glucose transport pathway based on studies where adenosine function was inhibited by either adenosine deaminase or various adenosine receptor antagonists (62, 437, 250). Therefore the involvement of adenosine in the synergistic mode of stimulation of muscle glucose uptake by insulin and by contractions is not entirely certain.

**Bradykinin and G\(_{i}\)-Coupled Receptors**

Bradykinin is a nonapeptide hormone that mediates physiological responses such as pain, inflammation and vascular permeability (329). During physical exercise, bradykinin is released
from many mammalian muscle types (381). Three classes of bradykinin receptors have been identified - B1, B2 and B3. Skeletal muscle and L6 muscle cells express B2 receptors (281) which couple to heterotrimeric G-proteins of the Gq class (256, 251). Ebina and colleagues showed that bradykinin directly triggers GLUT4 translocation by a PI3-K-independent (insulin-independent) pathway in L6 myotubes, 3T3-L1 adipocytes, and Chinese hamster ovary (CHO) cells expressing Gq-coupled bradykinin receptors (205). Furthermore, infusion of rats with a bradykinin receptor antagonist completely blocks exercise-stimulated glucose uptake and GLUT4 translocation to the plasma membrane of skeletal muscle cells (397). These results suggest that local release of bradykinin from contracting muscles may regulate GLUT4 translocation and glucose transport in skeletal muscles, although the link between Gq receptor activation and GLUT4 translocation is less clear. In contrast to the above findings, bradykinin has no effect on glucose transport in dog skeletal muscle or L6 myoblasts (281), but rather potentiates insulin-signalling (insulin receptor tyrosine kinase) and insulin-stimulated glucose uptake. Although exercise was not studied in the latter study, the results suggest that bradykinin feeds into the insulin-signalling pathway rather than participating in the alternative pathway. Thus, the participation of bradykinin in the contraction-dependent muscle glucose transport is a possibility that remains to be thoroughly investigated.

Ca2+-Calmodulin Dependent Kinase II
In addition to cPKC and NO, a rise in intracellular Ca2+ can activate other Ca2+-sensitive signalling molecules such as the serine/threonine kinase Ca2+-calmodulin dependent kinase II (CaMKII). CaMKII enzymes are expressed in every tissue and become activated and autophosphorylated when they bind calcium-saturated calmodulin. The kinase is believed to be linked to exocytosis through its ability to phosphorylate the synaptic vesicle protein synapsin I, and induce synaptic vesicle release from the cytoskeleton, an initial event in the exocytosis of
synaptic vesicles in neurons (32). CaMKII is involved in other regulated exocytic events such as the secretion of insulin from pancreatic cells (298). The involvement of CaMKII in regulated exocytosis lead to the examination of the role of CaMKII in the traffic of GLUT4 vesicles. To date, there is one report that a selective pharmacological inhibitor of CaMKII partially blocks the rise in muscle glucose transport stimulated by hypoxia in rats (47). However, in this study, the CaMKII inhibitor also partially inhibited insulin-stimulated glucose uptake. Because the hypoxia- or insulin-stimulated activation of CaMKII (and its inhibition by the drug) has not yet been shown, more conclusive evidence is required to determine whether CaMKII regulates GLUT4 translocation, and whether the enzyme represents a point of convergence of the two glucose transport pathways.

A current model summarizing the role of the above intermediaries in the glucose transport pathway stimulated by metabolic demand is presented in Figure 1.4. Also presented are the mediators of the insulin-dependent glucose transport system, which are reviewed in the next section.

**Insulin-Dependent Glucose Transport Regulation**

The anabolic hormone insulin is best recognized for its promotion of glucose uptake into skeletal muscle and fat for storage as glycogen and conversion into fat, although it regulates several other cellular responses including mitogenesis and antilipolysis. As described above, the effective removal of glucose from the circulation after a meal results from the insulin-stimulated translocation of preformed glucose transporters to the cell surface to increase glucose flux. This phenomenon summons a complex machinery of steps, beginning with signal transduction through kinases that utilize ATP and ending with the mobilization of GLUT4 (and GLUT1) from intracellular storage sites to the plasma membrane, where fusion and functional
incorporation with the cell surface occurs. In isolated cells or cells in culture (muscle and fat lines), this translocation phenomenon occurs within minutes of exposure to insulin. To date, the exact molecular mechanism by which insulin induces the translocation of GLUT4 to the plasma membrane is not fully understood, although many of the proximal signalling events have been mapped. Below is a summary of the current knowledge of the complex signal transduction cascade activated by insulin in muscle and fat cells.

Figure 1.4 Current model of insulin-dependent and -independent GLUT4 translocation
Alternative Pathway: The decrease in ATP levels during contractions or hypoxia results in Ca^{2+} release from the SR which triggers the activation of a variety of putative mediators including, cPKC, AMPK, and NOS. The production of NO, bradykinin, and adenosine during contractions, together with activated cPKC and AMPK somehow triggers the translocation of a selective intracellular pool of GLUT4 (and GLUT1) to the plasma membrane. **Insulin-Dependent Pathway:** Insulin receptor activation binds to and activates IRS which stimulates the activity of PI3-K, leading to an increase in the membrane concentration PI-3,4,5-P_3. PDK and Akt interact with PI-3,4,5-P_3 through their PH domains whereas aPKC bind to the membrane via other domain interactions. The interaction of Akt and aPKC with the phospholipid(s) alters their conformation so that they become accessible for phosphorylation and activation by PDK1 and PDK2. Activation of Akt and aPKC then stimulates GLUT4 translocation to the cell surface by a currently unknown mechanism. Adapted from Khayat, Patel and Klip (Can. J. Appl. Physiol., 2001).

The Insulin Receptor (IR)

The insulin receptor (IR) is an α_3β_2 heterotetramer. The α-subunits (135 kDa) are linked to each other and to the β-subunits (95 kDa) by disulfide bonds, and are located exclusively on the extracellular portion of the plasma membrane where they contain the insulin binding site. The β-subunits span the membrane and possess several functional regions including the tyrosine kinase domain (contains the ATP-binding site) and the juxtamembrane region, which are important for the biological actions of the receptor.

As summarized in Figure 1.4, the insulin signalling cascade begins with occupation of the IR by the hormone. This leads to activation of the tyrosine kinase domain, the first biochemical consequence of the hormone binding (267, 189). The activated tyrosine kinase then autophosphorylates on several tyrosine residues within the β-subunit juxtamembrane region and carboxy-terminus [reviewed in (459)], and phosphorylates several cellular substrates which propagate the insulin signal. Currently, these substrates include a family of proteins referred to as insulin receptor substrates (IRS, described in detail below). Substrate selection is mediated by the juxtamembrane region of the IR which engages the phosphotyrosine binding (PTB) domain in the IRS-proteins via the tyrosine autophosphorylation motif NPXY_α60. The tyrosine kinase activity of the IR is essential for insulin action as point mutations in the ATP-binding domain abolish insulin signalling (70).
The application of gene knockout technology in recent years has provided insight on the participation of mediators of insulin signalling in glucose homeostasis. Inactivation of the IR gene in mice leads to major metabolic defects and death of IR<sup>−/−</sup> pups within 1 week after birth (176), as expected. Surprisingly, mice with specific knockout of the insulin receptor in muscle, display normal blood glucose and insulin levels, and normal glucose tolerance despite impaired insulin-stimulated glucose uptake in skeletal muscle (48). This unprecedented finding suggests that tissues other than muscle may be more involved in insulin-regulated glucose disposal than was previously recognized. Interestingly, although the muscle IR knockout mice have no glucose transport response to insulin, they exhibit normal exercise-stimulated glucose uptake, indicating that the alternative pathway remains intact in the absence of IR in the muscle.

**Insulin Receptor Substrate (IRS)**

IRS proteins become tyrosine phosphorylated on multiple sites upon binding to the activated insulin receptor. When phosphorylated, IRS proteins can serve as receptor specific docking proteins that recruit and engage multiple signalling proteins to their phosphorylation sites. To date, four IRS isoforms have been identified, designated IRS1-4, which provide an expanded repertoire of signalling pathways for regulation by insulin. IRS-1 and IRS-2 are ubiquitously expressed (391, 392) while IRS-3 is highly expressed in adipocytes (242). It is unknown if IRS-4 is a physiologically relevant mediator of insulin action as it is mainly expressed in a human embryonic kidney (HEK) cell line (243). In addition to the PTB domain of IRS, which couples the protein to the insulin receptor, IRS proteins contain a well-conserved pleckstrin homology (PH) domain that binds membrane phospholipids and proteins and is also important for transmitting the insulin signal (478, 427).

The IRS proteins have a wide molecular mass range: IRS-1, 132 kDa (9); IRS-2, 190 kDa (411); IRS-3, 60 kDa (242); IRS-4, 160 kDa (243). The most well-characterized isoform.
IRS-1 serves as the prototype for this family of molecules. IRS-1 is normally a cytosolic protein containing 21 potential tyrosine phosphorylation sites including 6 in YMXM motifs, 3 in YXXM motifs, and 12 in other hydrophobic motifs (Y=Tyr, X=any amino acid and M=methionine) (459). At least 8 of these motifs undergo phosphorylation by the activated insulin receptor. In addition, IRS-1 contains over 30 potential serine/threonine phosphorylation sites in motifs recognized by a variety of kinases including casein kinase-2, mitogen-activated protein kinase (MAPK, see below), and protein kinase B (PKB, see below) (459). It has been proposed that serine phosphorylation by these kinases may be a mechanism of downregulating IRS-1 signalling (177).

IRS proteins propagate their signal by binding to and activating downstream signalling molecules containing src-homology 2 (SH2) domains. SH2 domains are protein modules 100 amino acids in length that recognize short phosphopeptide motifs such as the YXXM or YMXM motifs found within IRS proteins. PI3-K is one of the first SH2-domain containing proteins that was found to associate with tyrosine-phosphorylated IRS-1 and to be involved in insulin’s mthat are docked by IRS proteins include two phosphotyrosine phosphatases, SHPTP1 and SHPTP2, Ras GTPase-activating protein (Ras-GAP), and growth factor receptor-bound protein 2 (Grb2) (459).

It is not known with certainty which of the IRS proteins is involved in the stimulation of GLUT4 translocation by insulin. IRS-1 was initially thought to be the principal mediator of GLUT4 translocation and the stimulation of glucose transport based on the following observations: 1) Overexpression of IRS-1 in rat adipocytes increases basal GLUT4 content in the plasma membrane (327); 2) Ablation of IRS-1 with antisense ribozyme in rat adipocytes reduces insulin-sensitivity of GLUT4 translocation (326). However, the ability to translocate GLUT4 and achieve maximum stimulation of glucose transport by insulin is unaltered. In
contrast, several lines of evidence now indicate that IRS-1 may not play a crucial role in insulin-stimulated GLUT4 translocation. First, interfering with IRS-1 function by microinjecting anti-IRS-1 antibodies, PTB domain constructs or NPX-phosphotyrosine peptides into 3T3-L1 adipocytes does not affect insulin-stimulated GLUT4 translocation (283). Moreover, knockout mice lacking IRS-1 retain a significant capacity to regulate blood glucose levels in response to insulin and do not become diabetic (10).

The existence of the homologous IRS-2 protein may explain the inconsistency of results regarding the participation of IRS-1 in insulin-stimulated glucose transport. Indeed, IRS-2 has recently emerged as the insulin receptor substrate most likely to mediate insulin-stimulated glucose transport because, unlike IRS-1, genetic ablation of IRS-2 causes diabetes in mice (465). However, it was subsequently shown that insulin-stimulated glucose uptake remains intact in adipocytes and skeletal muscle of IRS-2 knockout mice (159). Additional evidence supporting a role for IRS-2 in insulin action comes from studies showing that overexpression of IRS-2 in rat adipocytes stimulates the translocation of GLUT4 in the absence of insulin (487). Although IRS-2 and IRS-1 have highly conserved PH and PTB domains, IRS-2 possesses an extra region between amino acids 591 and 786, which binds to the regulatory loop of the insulin receptor (353). Designated the kinase regulatory loop binding domain (KRLB), this novel region may contribute to a unique signalling potential for IRS-2.

The IRS-3 subtype is also highly tyrosine phosphorylated by the insulin receptor and contains the same overall architecture of IRS-1 and IRS-2. Some researchers propose that IRS-3 is important in regulating insulin action in fat cells because in adipocytes derived from IRS-1-null mice, PI3-K associates more rapidly with IRS-3 than IRS-2 (369). However, mice deficient in IRS-3 show normal glucose tolerance and normal insulin levels (253), suggesting that IRS-3 is not essential for glucose homeostasis.
Which of the four isoforms of IRS proteins participate(s) in the stimulation of glucose transport is not certain at present. All proteins in the family are reasonable candidates because they are all able to engage the key signaling molecule important for insulin-stimulated glucose transport stimulation, PI3-K (see below).

**Phosphatidylinositol 3-Kinase (PI3-K)**

As alluded to in earlier sections, the central enzyme in the insulin signalling pathway towards glucose metabolism is PI3-K (225), an enzyme that possesses dual lipid and protein serine kinase activity (361) and interacts with all the known IRS proteins (460). *In vitro*, PI3-K phosphorylates the 3'-OH of the inositol ring of phosphatidylinositol phospholipids (Figure 1.5) to generate phosphatidylinositol-3 phosphate (PI3-P), PI-3.4-bisphosphate (PI-3.4-P₂) and PI 3,4,5-trisphosphate (PI-3.4.5-P₃). The preferred PI3-K substrate *in vivo* is PI-4.5-P₂ (361); thus the PI-3.4.5-P₃ product is the key lipid mediator of intracellular insulin signalling. The rise in phosphorylated phospholipid products caused by PI3-K activation *in vivo* is transient (t₁/₂ ~ 5 min) (83), yet sufficient to allow PI-3,4,5-P₃ to bind to PH domains in a variety of proteins, affecting their membrane localization, conformation, and activity. The downstream effectors of PI3-K relevant to insulin-dependent GLUT4 translocation are the serine/threonine kinases Akt (protein kinase B, PKB) and aPKCs. PKCλ and PKCζ (discussed in detail further on).

*Figure 1.5 Biological action of PI3-K*
The PI3-K Family of Enzymes

The multiple isoforms of PI3-K are organized into three classes: class I, class II, and class III. The PI3-K isoforms within each class are summarized in Table 1.3. All catalytic subunits of all PI3-K contain the kinase domain at their carboxyl terminus preceded by the PIK (PI-kinase homology domain). Class I PI3-K are heterodimers made up of an 85-kDa regulatory subunit (p85) that is likely constitutively associated with a 110-kDa catalytic subunit (p110) (99) and allows for preferential phosphorylation of PI-4,5-P$_2$ in vivo. This class of PI3-K is further subdivided into class IA and class IB which signal downstream of tyrosine kinases and heterotrimeric G-protein coupled receptors, respectively. Class I PI3-Ks are normally cytosolic in quiescent conditions and associate with membranes when cells are stimulated.

Class II PI3-K are large molecules (>170 kDa) whose distinguishing feature is a carboxy-terminal C2 domain which binds phospholipids in vitro in a Ca$^{2+}$-independent manner (361). In contrast to class I PI3-K, the substrate preference of class II enzymes is PI$>$PI-4-P$>$PI-4,5-P$_2$ and these enzymes are predominantly associated with cell membranes in the absence of any stimulation (11, 440).

<table>
<thead>
<tr>
<th>Table 1.3 Classes of mammalian PI3-K</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CLASS</strong></td>
</tr>
<tr>
<td>IA</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>IB</td>
</tr>
<tr>
<td>II</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>III</td>
</tr>
</tbody>
</table>
The third class of PI3-K consists of enzymes which are homologues of the yeast vacuolar protein sorting protein Vps34p (356). In vitro and in vivo, the major product of these class III PI3-K is PI3-P (433), suggesting that these enzymes are “housekeepers” which maintain the cellular pool of PI3-P required for constitutive membrane traffic and vesicle morphogenesis (95).

Although insulin is able to stimulate enzymes in the different classes of PI3-K, it is currently thought that the type IA PI3-K enzyme participates in insulin-dependent mobilization of GLUT4 to the plasma membrane, and glucose uptake (359). Indeed, insulin stimulates class IA PI3-kinases in a similar time frame and with similar dose-dependence to its stimulation of glucose transport (195). As mentioned above, the type IA PI3-K consists of a p85 regulatory and a p110 catalytic subunit. Following insulin treatment, the two SH2 domains of p85 interact with phosphotyrosine residues within a YMXM or YXXM motif (see above) on the IRS molecules. This results in a marked increase in the lipid kinase activity of the associated 110 kDa catalytic subunit. The p85-p110 complex is normally cytosolic, but it relocates in response to insulin: within minutes of hormonal stimulation, p85 and p110 associated with tyrosine-phosphorylated IRS segregates with the intracellular membranes (low density microsomes, LDM) isolated from rat adipocytes and 3T3-L1 adipocytes (196, 195). This fraction represents a subpopulation of internal membranes from which the intracellular pools of glucose transporters are isolated. It is thought that this relocalization of PI3-K to the LDM allows PI3-K lipid products to bring downstream signalling effectors into close proximity with GLUT4-containing compartments to allow for their exocytosis. This phenomenon is explored in detail in the results presented in Chapter 3.

To date, three isoforms of the class IA catalytic subunits have been identified: only two (p110α and p110β) are believed to participate in insulin signal transduction (432). Of the five
regulatory subunits that couple to the class IA catalytic subunits (p85α, p85β, p50α, p55α and p55γ) (99, 8, 325), it is currently believed that only p85α is important for the translocation of glucose transporters and the stimulation of glucose transport by insulin (see below).

**Discerning the Role of PI3-K in Insulin Action**

The fungal metabolite wortmannin (Figure 1.6) has facilitated the discovery of downstream consequences of PI3-K activation (426). Wortmannin irreversibly inhibits PI3-K by covalently binding to all 110 kDa catalytic subunits, thereby obliterating PI3-K activity. Although wortmannin has been reported to inhibit the activity of another phospholipid kinase, PI4-K. in cells in culture, inhibition of this enzyme requires much higher concentrations (~1 μM) of wortmannin than is required for full PI3-K inhibition (<100 nM). Wortmannin provided the initial insights that PI3-K was one of the long sought after mediators of insulin-stimulated glucose transport. The inhibitor blocks the stimulation of glucose transport and the translocation of GLUT1 and GLUT4 in rat skeletal muscle (477), adult human skeletal muscle (360), L6 muscle cells (417), rat adipocytes (308), and 3T3-L1 adipocytes (74). Because wortmannin does not alter the initial endocytosis (internalization) of GLUT4 (or GLUT1) from the plasma membrane (475) it could be reasoned that PI3-K mediates the exit of glucose transporters from intracellular compartments, but not their reentry into endosomes. The results obtained using wortmannin in 3T3-L1 adipocytes and skeletal muscle have also been confirmed using LY294002 (64, 360), a structurally distinct selective inhibitor of PI3-K (441).

**Figure 1.6 Structural features of the PI3-K inhibitor wortmannin**

![Figure 1.6 Structural features of the PI3-K inhibitor wortmannin](image-url)
Besides the pharmacological approach, several other strategies have been used to show that PI3-K participates in the stimulation of GLUT4 translocation and glucose transport by insulin: 1) Overexpression of either the p110α catalytic subunit of PI3-K (190) or a constitutively active form of p110 (406) stimulates basal PI3-K activity and increases glucose transport and the redistribution of GLUT1 and GLUT4 to the cell surface, albeit not to the same extent as insulin: 2) Overexpression of a dominant negative mutant of p85α lacking its SH2 domain, which is unable to bind and therefore activate the catalytic p110 subunit, prevents the stimulation of glucose transport by insulin (225, 328); 3) Microinjection of a mutant p85α or a (glutathione S-transferase) GST-p85 fusion protein interferes with the normal insulin-induced translocation of GLUT4 in 3T3-L1 adipocytes (149); 4) There is an increase in PI3-K activity in intracellular GLUT4-containing compartments a few minutes after insulin stimulation in 3T3-L1 adipocytes (153, 453); 5) Overexpression of the lipid phosphatases PTEN (phosphatase and tensin homologue deleted from chromosome ten) and SHIP (SH2-containing inositol phosphatase), which remove the 3’ phosphate and the 5’ phosphate from PI-3,4,5-P3, respectively, diminishes PI-3,4,5-P3 levels and markedly reduces insulin-stimulated glucose uptake and GLUT4 translocation (292, 446). These findings have been instrumental to our understanding of the stimulation of glucose transport by insulin, in that they provide the first link between the insulin receptor/IRS and the mobilization of glucose transporters.

*Signals Acting Downstream of PI3-K: Akt/Protein Kinase B (PKB) and Atypical PKC (aPKC)*

It is well-established that PI3-K is the key mediator of the insulin signal transduction cascade. However, the function of the phospholipid products is not well understood. It is conceivable that they alter the curvature and other physical and chemical properties of the membrane by virtue of their additional negative charge (252), but this possibility remains untested. Whether the PI-3,4,5-P3 lipid product is actually generated on GLUT4-containing
endomembranes to promote vesiculation, membrane fusion or vesicle traffic is an intriguing possibility that has technically eluded scientists for years. Currently it is known, however, that PI3-Ps are cofactors required for the activation of other enzymes. The two major natural targets that are thought to carry the signal initiated by PI3-K activation to its final destination are the serine/threonine kinases Akt (or protein kinase B, PKB) and the aPKC proteins. PKC\(\alpha\) and PKC\(\zeta\).

Akt/Protein Kinase B (PKB)

Akt Family Members and their Mechanism of Activation

Akt, also known as protein kinase B (PKB), is a serine/threonine kinase homolog of the transforming oncogene viral Akt (v-Akt) (27). The catalytic domain of Akt is most similar to those of protein kinase A (PKA) and PKC: findings that gave rise to its three names, PKB, RACK (related to A and C kinases), and cAkt (80). In this Thesis, the enzyme will be referred to as Akt. To date, three 55-60 kDa Akt isoforms have been identified: Akt1,2 and 3 or PKB\(\alpha\), \(\beta\) and \(\gamma\) (81). All three proteins share similar structural features, including a PH domain in the amino-terminus, followed by a short glycine-rich region that bridges the PH domain and the catalytic domain. The last 70 amino acids of the carboxy-terminal tail contain a putative regulatory domain. All three Akt isotypes have conserved threonine and serine residues (Thr 308 and Ser 473 in Akt1: Thr 309 and Ser 474 in Akt2, and Thr 305 in Akt3) that together with the PH domain are crucial for Akt activation (see below). Akt1 and Akt2 are similar in size and are ubiquitously expressed (28), whereas Akt3 is highly expressed in brain, testis and all cells in culture (222). There are two splice variants of Akt3: one lacks a portion of the C-terminus containing the regulatory phosphorylation site (222); the other has a similar structure to Akt1 and 2 (293). Classically, Akt has been implicated in signalling by tyrosine kinase receptors to
promote cell survival and growth (66, 29). However, in the past 5 years Akt has been shown to be involved in insulin signalling towards glucose metabolism (see below).

Akt is activated by two upstream kinases – PI3-K and 3-phosphoinositide-dependent kinases (PDKs). The requirement for PI3-K in Akt activation has been well-documented based on pharmacological and molecular strategies interfering with PI3-K function (53, 122, 216, 30). These and other studies showed that two 3’phosphoinositol products of PI3-K (PI-3,4-P₂ and PI-3,4,5-P₃) bind with high affinity to the PH domain of Akt and may participate in activating the enzyme (216, 123, 124).

It was originally thought that the src myristoylation signal in Akt mediates targeting to the plasma membrane where it binds PI-3,4-P₂/PI-3,4,5-P₃ and becomes fully activated. It is now appreciated that the primary mechanism of Akt activation on the membrane is via its phosphorylation on serine and threonine residues in the C-terminal regulatory domain (described above) (220). Mutation of these amino acids to nonphosphorylatable residues abolishes kinase activation. whereas mutations to acidic residues render Akt constitutively active (30). Akt phosphorylation is achieved by PDK, a kinase whose activity is dependent on PI3-K-dependent generation of PI-3,4-P₂ and PI-3,4,5-P₃ in the membrane (7, 384). PDK1 threonine phosphorylates Akt (residue 308) (6). and PDK2 (a so-far unidentified kinase) putatively phosphorylates serine 473 on Akt (7). Although the molecular identity of PDK2 is not yet known. it has been suggested that PDK1 and PDK2 might constitute the same enzyme that can phosphorylate both residues of Akt in vivo (15). PDK1 has a carboxy-terminal PH domain which binds with high affinity to PI-3,4,5-P₃ and more weakly to PI-3,4-P₂. It is currently thought that PDK enzymes are always active, and a small portion of the enzyme is always found in the plasma membrane due to its high affinity for PI-3,4,5-P₃. Local generation of PI-3,4-P₂ and PI-3,4,5-P₃, that results from PI3-K activation by insulin is thought to recruit
Akt's PH domain to the membrane where it becomes accessible to phosphorylation by membrane-bound PDK1 and PDK2 (Figure 1.4).

**Role of Akt in Insulin Action**

All three Akt isoforms are differentially regulated by insulin in a tissue-specific manner. In skeletal muscle, insulin activates all Akt isotypes (422), although Akt1 is thought to be the predominant isoform activated (448). In isolated rat adipocytes, Akt2 is the major insulin-responsive isoform (448). Akt3 is mainly activated by insulin in cultured cell lines such as L6 muscle cells and 3T3-L1 adipocytes (448). In L6 muscle cells, Akt1 and Akt2 are activated to similar extents (448), albeit less than Akt3, whereas all three isoforms are activated equally by insulin in 3T3-L1 adipocytes in culture (394).

Activation of all Akt isoforms by insulin is contingent upon prior activation of PI3-K, regardless of the cellular background. This realization led to the suggestion that Akt may be a protein kinase that functions downstream of PI3-K in the regulation of glucose uptake by insulin. Indeed, a constitutively active Akt1 mutant, generated by adding a membrane targeting motif (Src myristoylation sequence) to various Akt1 constructs, is sufficient to elicit maximal stimulation of glucose uptake and translocation of GLUT4 in 3T3-L1 adipocytes, independently of insulin (219). In addition, overexpression of wild-type or constitutively active Akt1 mutants elevates GLUT4 translocation in L6 muscle cells (424), rat adipocytes (82) and 3T3-L1 adipocytes (407, 418). Although these studies suggest that activation of Akt may be sufficient to stimulate GLUT4 translocation to the extent achieved by insulin, they do not prove that Akt1 is necessary for the response. Microinjection of an Akt2 substrate peptide or an antibody to Akt2 was shown to reduce more than half of the insulin-stimulated GLUT4 translocation to the plasma membrane of 3T3-L1 adipocytes (161). Additionally, Akt mutants that may act as dominant negative inhibitors of endogenous Akt have been used to discern the role of this
enzyme in insulin action. The sensitivity of insulin-induced GLUT4 translocation is reduced in rat adipocytes transfected with a kinase-inactive mutant of Akt1 (82). Furthermore, Akt1 responsiveness is inhibited by 70% in L6 muscle cells overexpressing a dominant negative Akt mutant (kinase dead and inactivatable) (452). This mutant (AAA-Akt) completely inhibits the endogenous Akt1 and Akt2 [(452), and Somwar and Klip. unpublished observations]. In contrast to these findings, overexpression of various Akt mutants in L6 myotubes and 3T3-L1 adipocytes, does not affect insulin-induced GLUT4 translocation (207, 19). Recent reports of a newly developed specific inhibitor of Akt (ML-9), and an Akt2 knockout mouse model have provided compelling evidence of the requirement for Akt in insulin action. In these studies, interfering with Akt by either approach significantly inhibits insulin-stimulated glucose uptake in brown adipocytes (156) and skeletal muscle (68), respectively.

\( \text{aPKC (PKC} \lambda \text{ and PKC} \zeta \)\)

\( \text{aPKC Isoforms and their Mechanism of Activation} \)

Atypical PKC isozymes (\( \lambda, \zeta, \iota \)) are distinct from all other members of the PKC family in that they are not activated by DAG or phorbol esters (see PKC section above). Rather. there is substantial evidence that PI3-K contributes to the activation of PKC\( \lambda \) and PKC\( \zeta \), by virtue of aPKC sensitivity to acidic phospholipids. In accord with this possibility, the insulin-stimulated activation of aPKC\( \zeta \) is blocked by PI3-K inhibitors (18, 378, 227) and is lost in cells expressing IRS-1 mutants lacking PI3-K-binding (273).

As with Akt activation, aPKC is thought to be activated via both phospholipid binding and phosphorylation of regulatory sites when the enzyme is bound to the membrane. In addition to autophosphorylation, PDK1 and PDK2 bind and putatively phosphorylate and activate aPKC (246, 71), although the latter possibility has not been formally tested. Another mechanism of activation of aPKC is by binding to PI3-K products in the membrane. The aPKC enzymes do
not contain PH domains, and the exact mechanism of phospholipid activation is not known. although it is clear that aPKC binds PI-3.4.5-P, with high affinity and that this contributes to its activation (291).

*Role of aPKC in Insulin Action*

It is well established that insulin rapidly activates aPKC isozymes in all cell types studied (18, 227, 17, 378). This activation of aPKC involves increases in both its enzyme activity and its autophosphorylation (378, 18, 379). Evidence suggesting a role for aPKC in insulin action came from studies showing that a PKCζ pseudosubstrate peptide, as well as other PKC inhibitors, provoke parallel dose-dependent decrements in the activity of PKCζ and insulin-stimulated glucose transport (378). Further evidence derives from transfection studies showing that expression of a constitutively active mutant of aPKC stimulates glucose uptake in quiescent cells, and a kinase-inactive form prevents insulin-stimulated glucose uptake and GLUT4 translocation in L6 muscle cells (18, 19) and 3T3-L1 adipocytes (227, 17). Similarly, expression of dominant-negative PKCζ inhibits translocation of GLUT4 to the plasma membrane in transiently transfected rat adipocytes in a manner that is reversible by wild-type PKCζ (378).

Taken together, the studies described above provide compelling evidence for a role of Akt and aPKC in insulin-induced GLUT4 translocation downstream of PI3-K, although the link between activation of these enzymes and GLUT4 mobilization to the cell surface has not been established. Clearly Akt and aPKC represent a bifurcation point in the insulin signalling cascade, although their downstream targets are unknown. Preliminary results show that certain isoforms of Akt and aPKC co-purify with immuno-isolated compartments containing GLUT4.
(56, 379, 236), suggesting that these enzymes may phosphorylate proteins on GLUT4 endomembranes or on the plasma membrane that are important for vesicle traffic or fusion.

Our current knowledge of the participation of all of the above signalling intermediaries in insulin-dependent GLUT4 externalization is summarized in Figure 1.4. In addition to triggering PI3-K-dependent GLUT4 mobilization, insulin also activates many other signalling cascades in muscle and fat cells which lead to a variety of cellular effects including glycogen synthesis, mitogenesis, and antilipolysis [all reviewed in (399)]. These other actions of insulin in target tissues will not be discussed further in this Thesis.

Other Insulin Signalling Pathways Implicated in GLUT4 Traffic

Besides the PI3-K→Akt/aPKC axis of insulin signalling towards GLUT4 translocation, several exciting potential mediators of the insulin response have recently emerged. Although these signalling intermediaries are not explored in the work presented in this Thesis, the following section provides a brief summary of the evidence implicating these pathways in insulin-stimulated glucose uptake.

General Receptor for Phosphoinositides-1 (GRP1), ARF6 and Phospholipase D (PLD)

In addition to directly activating downstream kinases such as Akt and aPKC, polyphosphoinositides generated by PI3-K are also thought to activate a class of proteins including general receptor for phosphoinositides-1 (GRP1/"ARNO3"), which contain both PH domains and Sec7 guanine nucleotide exchange domains for ADP-ribosylation factor (ARF) proteins (405, 241). Insulin stimulation causes GRP1 and the small G-protein ARF6 to translocate to the plasma membrane (436, 188), where ARF6 is activated, thereby leading to the activation of phospholipase D (PLD). Whether ARF6 activation is required for insulin action is controversial because several studies showed that overexpression of mutant forms of ARF6 does
not affect basal or insulin-regulated GLUT4 translocation in 3T3-L1 adipocytes (473, 307, 245, 42), whereas another study showed ARF6 to be important for this response (276). However, the downstream ARF6 effector PLD does colocalize with intracellular GLUT4-containing compartments (275) and is able to potentiate the effects of insulin on GLUT4 translocation (111). It has been proposed that PLD on membranes produces other lipid mediators which may in turn activate downstream kinases that somehow regulate GLUT4 translocation (377, 105).

**PI3-K-Independent Mediators of Insulin-Stimulated GLUT4 Translocation**

Although the requirement for PI3-K activation in insulin-stimulated GLUT4 translocation is indisputable, recent evidence suggests that activation of the enzyme alone is not sufficient to elicit maximal GLUT4 externalization in response to insulin (174, 142, 169). The search for PI3-K-independent mediators of insulin action has uncovered two attractive cellular pathways which may contribute to the full translocation of GLUT4 to the cell surface in response to insulin.

**Heterotrimeric GTP-Binding Proteins**

Pertussis toxin (PTX) is known to suppress the function of heterotrimeric GTP-binding proteins (338). Pretreatment of isolated rat soleus muscle or adipocytes with PTX causes a dose-dependent decrease in insulin-stimulated glucose uptake (183). Moreover insulin induces tyrosine phosphorylation of the 40 kDa α-subunit of G_i (α_3), suggesting that G_i may participate in insulin action in these tissues. However, in 3T3-L1 adipocytes, overexpression of neither the wild-type nor constitutively active forms of G_i and G_s α-subunit affects basal or insulin-stimulated translocation of a co-expressed GLUT4-enhanced green fluorescent protein (EGFP) fusion protein. On the other hand, expression of a constitutively active G_{as,11} mutant causes GLUT4 translocation in the absence of insulin, and in a wortmannin-insensitive manner (245, 185, 42). Furthermore, microinjection of an α-subunit-specific antibody inhibitory to G_q or G_{11}
partially blocks GLUT4 translocation (168, 185). These data are consistent with the hypothesis that insulin stimulation of GLUT4 translocation involves trimeric GTP-binding proteins.

**CAP/Cbl/Flotillin/TC10 Signalling Pathway**

A new signalling pathway triggered by insulin has been proposed to occur in segregated compartments of the plasma membrane called caveolae. These are small invaginations of the membrane enriched in lipid-modified signalling proteins, glycolipids, sphingolipids, and cholesterol (368). A complex of the protooncogene c-Cbl and Cbl-interacting protein (CAP) constitutively binds to the insulin receptor (IR) in quiescent insulin-responsive cells (339). Upon insulin stimulation, Cbl is phosphorylated by the IR, causing release of the complex from IR and its accumulation in caveolae (264) via association with the caveolar protein flotilllin (24). Phosphorylated Cbl in the caveolae then recruits SH2-containing proteins including CrkII which recruits C3G, an exchange factor for the small molecular weight GTP-binding protein TC10 (tetracarcinoma clone 10, see below) (109) to the complex. Evidence for an involvement of this signalling complex in insulin action is derived from three major observations in 3T3-L1 adipocytes: 1) Overexpression of a dominant negative (delta SH3) mutant of CAP reduces insulin-stimulated GLUT4 translocation and glucose uptake (24); 2) TC10 is expressed in fat and muscle cells, and in 3T3-L1 adipocytes heterologously expressed TC10 can be rapidly activated by insulin in a CAP-dependent, but PI3-K-independent manner (67); 3) Overexpression of either wild-type, constitutively active, or dominant negative TC10 into 3T3-L1 adipocytes reduces insulin-stimulated GLUT4 translocation and glucose uptake (67). Although the exact function of TC10 is unknown, the current hypothesis is that the Rho family protein regulates processes involved in actin cytoskeleton remodeling or GLUT4 vesicle fusion (67).
REGULATION OF GLUT4 VESICLE DOCKING AND FUSION – THE SNARE HYPOTHESIS

The externalization of GLUT4-containing compartments with the plasma membrane in response to insulin represents a phenomenon of regulated exocytosis: the controlled incorporation of an intracellular compartment (e.g. GLUT4 endomembranes) with the cell surface, in response to a stimulus (e.g. insulin). The event therefore includes generation of the signal by the stimulus, detection of the signal by the exocytic compartment, recognition of the target site on the plasma membrane, and fusion of the lipid bilayers of the incoming vesicle compartment and the cell membrane.

Most of the information available on the steps involved in regulated vesicle traffic is based on studies of neurotransmitter exocytosis. This is a highly specialized form of regulated exocytosis involving additional aspects of vesicle sorting, vesicle priming, fusion, re-endocytosis and refilling. Significantly, proteins of the SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) family participate in the early stages of this process (372, 371). The SNARE hypothesis originally suggested that specific proteins on the vesicles (v-SNAREs) interact with specific proteins on their target membranes (t-SNAREs) to dock the vesicle, and to promote fusion between the two membranes (Figure 1.7). More recent models ascribe to the SNARE molecules a role in fusion but not necessarily in docking (457). The original suggestion that specific pairing between SNARE proteins is responsible for maintaining targeting fidelity (372) has been replaced by the concept that other proteins are involved in mediating recognition and docking specificity. Nevertheless, it is clear that v- and t-SNAREs play an essential role in regulated exocytosis in all systems in which they have been tested, and form a template for a potentially universal mechanism of vesicle recognition leading to membrane fusion.
Despite obvious morphological and physiological differences between synaptic vesicle exocytosis and insulin-dependent externalization of glucose transporters, the two processes show several similarities. Molecular cloning of the brain SNARE cDNAs led to the discovery of other SNARE isoforms expressed in insulin responsive tissues (330, 445, 312, 444, 443, 387, 335, 466, 337), confirming that the SNARE hypothesis presents a general model of vesicle traffic events. Like the neuronal syntaxin-1 and SNAP25, the more ubiquitous isoforms syntaxin-4 and SNAP-23 are predominantly on the plasma membrane (312, 445), whereas VAMP2/synaptobrevin and VAMP3/cellubrevin are associated with intracellular membranes including those containing GLUT4 (54, 312, 443, 444). Like their neuronal counterparts, they bind in all combinations of binary complexes with high affinity, although they may not form as stable ternary complexes (119).

There is strong functional evidence supporting the idea that the incorporation of insulin-responsive GLUT4 containing vesicles into the plasma membrane of muscle and adipose cells requires the v-SNARE protein isoform VAMP2 (but not the related VAMP3/cellubrevin isoform), and the t-SNAREs syntaxin-4 and SNAP-23. Introduction of Clostridial (tetanus) neurotoxins [which specifically cleave VAMP2 and VAMP3 (37)] into 3T3-L1 adipocytes abrogates GLUT4 arrival into the plasma membrane in insulin-stimulated cells (65, 403, 257). It was later shown in L6 muscle cells that the inhibition of GLUT4 translocation caused by tetanus toxin could be rescued by transfecting a toxin-resistant VAMP2 mutant, but not by a similar VAMP3 mutant (333). Introduction of peptides comprising various regions of VAMP2 or syntaxin-4, as well as polyclonal antibodies raised against the proteins have been shown to inhibit GLUT4 translocation and/or glucose transport (445, 312). Recombinant, soluble forms of these proteins, when introduced into or expressed in 3T3-L1 cells also prevent GLUT4 arrival, presumably by competing for binding partners of the endogenous, membrane-bound
proteins (445, 312, 257). Introducing deletion mutants or neutralizing antibodies to SNAP-23 into 3T3-L1 adipocytes reduces GLUT4 arrival at the plasma membrane (120, 337, 192) and insulin-dependent glucose uptake (120). Finally, insulin-stimulated GLUT4 translocation and glucose uptake is reduced by 50% in skeletal muscle of syntaxin 4-knockout mice (471). Taken together, these studies strongly support a participation of VAMP2, syntaxin-4 and SNAP-23 in insulin stimulated GLUT4 traffic and glucose transport.

**Figure 1.7 The SNARE hypothesis in GLUT4 traffic**

On the plasma membrane of insulin-stimulated cells, the t-SNAREs, SNAP-23 and syntaxin-4 form a complex with the v-SNARE VAMP2 associated with GLUT4 vesicles (G4). Dissociation of munc-18C and VAP33 from syntaxin-4 and VAMP2, respectively, may prime the vesicle for docking to the plasma membrane. The energy derived from this 'SNARE' complex assembly drives the subsequent bilayer fusion of the GLUT4-containing vesicle with the plasma membrane. SNARE complexes then dissociate, perhaps aided by N-ethyl maleimide sensitive factor (NSF), for a new cycle. Adapted from Foster, Khayat and Klip, *Diabetes Annual*, 1999.
As in nerve terminals, binding partners of the SNARE proteins are likely to modulate GLUT4 incorporation into the plasma membrane. Synip and Munc-18c, which bind syntaxin-4, prevent GLUT4 arrival at the membrane when overexpressed in 3T3-L1 adipocytes (404, 277). Similarly, overexpression of the VAMP partner VAP33 impairs insulin-stimulated GLUT4 recruitment to the plasma membrane of L6 muscle cells (421). These results are consistent with the important role for SNARE proteins in insulin-dependent GLUT4 insertion into the membrane in insulin-responsive tissues. Figure 1.7 presents a model for SNARE-dependent GLUT4 incorporation into the plasma membrane.

Although much is known about SNAREs in insulin-regulated GLUT4 traffic, studies implicating SNARE proteins in contraction-dependent GLUT4 externalization are lacking. It is known that, like insulin, contraction stimulates the redistribution of VAMP2 from an intracellular site to the plasma membrane, along with GLUT4 (231), although the functional requirement for VAMP2 in the exercise response has not been determined. The syntaxin-4 binding partner, Munc18c, is required for contraction- and insulin-dependent GLUT4 movement to the T-tubules in skeletal muscle (199), suggesting (but not proving) that syntaxin-4 may be important for exercise-regulated GLUT4 traffic. Finally, mice lacking the VAMP3 gene exhibit normal exercise- and insulin-stimulated GLUT4 traffic (472), consistent with previous findings with insulin in cells in culture (333). It is likely then that similar SNARE proteins regulate GLUT4 vesicle mobilization in the alternative pathway, although this hypothesis still requires extensive testing.

PARTICIPATION OF THE CYTOSKELETON IN INSULIN-DEPENDENT GLUCOSE TRANSPORTER TRANSLOCATION

The need for the cytoskeleton in regulated membrane traffic is becoming evident. Cytoskeletal components form a dense network which extends throughout the cytoplasm of all eukaryotic cells and coordinates cell movements. These movements include the maintenance of
cell shape, crawling on a substratum, muscle contraction, movement of cilia, and intracellular positioning and movement of membrane vesicles and organelles. The three main cytoskeletal networks that perform these functions in eukaryotic cells are the actin or microfilament network, the tubulin or microtubule network, and the intermediate filament network. The following section summarizes the current knowledge of the three cytoskeleton systems in mammalian cells including the evidence for the participation of these systems in insulin-stimulated glucose transporter translocation. Emphasis is placed on the actin-based cytoskeleton because it comprises the majority of the research presented in this Thesis.

The Actin Network

Actin Structure and Morphology

Actins are small globular 42 kDa polypeptides consisting of about 375 amino acids. Mammalian cells express at least three different actin genes: α, β, and γ (94). Alpha actin is also known as sarcomeric actin because of its almost exclusive expression and participation in myofilament contraction within the sarcomeres of cardiac and skeletal muscle (385). There are three types of α-actin, α-skeletal, α-cardiac, and α-smooth muscle. The β and γ actin isoforms are ubiquitously expressed and are responsible for all other motility functions ascribed to actin, except for contraction. The properties of β and γ actin isolated from mammalian skeletal muscles are common to actin from all other sources, which points out the significance of this protein for cellular function (178). In most mammalian cells, non-sarcomeric actin assembles into filaments (see below) that form bundles (also called stress fibers) running through the cytoplasm. Figure 1.8 shows the typical appearance of the F-actin stress fibers of L6 myotubes.

In its monomeric form, actin is called G (globular) actin, and is noncovalently associated with an ATP molecule. In vivo, G actin spontaneously polymerizes into F (filamentous) actin by a process involving hydrolysis of the bound ATP to ADP. Each filament of actin has a
polarity that is important for its function, consisting of a fast growing end (also termed the plus or barbed end because it resembles the barb of an arrow when actin is decorated with myosin), and a slow growing end (also called the minus or pointed end because it resembles the point of an arrow). The majority of filamentous growth occurs at the plus end normally directed towards the plasma membrane of cells, while the minus filament end is directed towards the cell interior (178). The assembly and disassembly of actin filaments is a dynamic process that continuously occurs in mammalian cells (depicted in Figure 1.9 in the section below).

**Figure 1.8 F-actin morphology of L6 skeletal muscle myotubes**

L6 myotubes grown on coverslips were fixed with 3% (vol/vol) paraformaldehyde and F-actin was stained using rhodamine-conjugated phalloidin. Cells were photographed with a fluorescent microscope (see Materials and Methods for details). Shown are the F-actin stress fibers running longitudinally along the myotube axis.

Actin and its associated proteins provide a matrix under the plasma membrane that not only supports cell shape, but also dynamically participates in many membrane processes such as phagocytosis, cytokinesis, locomotion, transmembrane signaling, endocytosis, and secretion [reviewed in (45)]. The membrane skeleton of the red blood cell has provided detailed insight into the arrangement of the cortical actin cytoskeleton. Isoforms and structures of proteins within the spectrin-actin network of erythrocytes occur adjacent to the plasma membrane of all higher cells, where they play different roles [reviewed in (33)]. These diverse cellular functions result from the rapid adaption of cortical actin to changing conditions such as those triggered by environmental stress or extracellular signals (354).
Role of Rho family GTPases in Cortical Actin Cytoskeleton Remodelling

The formation and reorganization of cortical actin during dynamic cellular responses are controlled by small molecular weight GTP binding proteins (G-proteins) of the Rho family, a subfamily of the Ras superfamily of small G-proteins (21). The Rho family has 16 members consisting of ten highly related Rho proteins (RhoA-E, RhoG, RhoH, Rho6-8), three Rac proteins (Rac-1, Rac-2, Rac-3), Cdc42, TC10 (described above), and TC10-like protein (TCL) [reviewed in (401)].

Whereas the actin microfilament changes elicited by TC10 and TCL are not clear. Rho, Rac and Cdc42 cause distinctive changes in the actin cytoskeleton in all cell types examined. Rho controls formation of stress fibers in response to growth factors and lysophosphatidic acid (342, 280). Stress fibers are the bundles of actin filaments that span the cell (Figure 1.8) and link to the extracellular matrix through focal adhesions. Rac proteins mediate the assembly of cortical actin filaments to produce lamellipodia and membrane ruffles in response to a variety of stimuli (343). Lamellipodia are thin protrusive actin sheets that dominate the edges of many migrating cells: membrane ruffles are observed at the leading edge cells and result from lamellipodia that lift up off the substratum and fold backward. Cdc42 has homology to Rac proteins, but is more important in the control of cell polarity and the formation of filopodia (305, 228). These structures are fingerlike protrusions that contain a tight bundle of long actin filaments in the direction of the protrusion, typically observed in motile cells.

The regulation of actin polymerization by the Rho family proteins requires their activation by extracellular stimuli. Like other Ras-related proteins, Rho family proteins are inactive in the GDP-bound state and assume an active conformation when bound to GTP. The GDP-to-GTP exchange results from an upstream signal leading to the activation of a family of over 15 guanine nucleotide exchange factors (GEFs, reviewed in (396)). Downstream of Rho protein activation, some 10 GTPase-activating proteins (GAPs) and 3 guanine nucleotide
dissociation inhibitors (GDIs) down-regulate the GTPase activity, but little is known about their mechanism of action (429). Mutations of Rho family proteins that lock into either the GDP- or GTP-bound state have become invaluable tools for elucidating their physiological roles. Mutation of Gly 14 of Rho family proteins to Val (similar to Val 12 mutation in Ras) inhibits GTPase activity, making the proteins unresponsive to its GAP and constitutively active (342). Mutation of Ser 17 to Asn (NI71 makes Rho family members unable to bind GTP to become activated, but presumably still able to bind activators, turning the proteins into dominant negative inhibitors when overexpressed (343).

The exact biochemical mechanism by which Rho family proteins cause actin remodeling is still under intense debate, and is beyond the scope of this Thesis. However, it is known that the processes involve several cellular effectors.

**Biological Tools to Study Actin Function**

Several cell-permeant, structurally unrelated compounds that disrupt the cytoskeleton are currently available, including cytochalasin D, latrunculin B, jasplakinolide, and swinholide-A (375) (Figure 1.9). The mold metabolite cytochalasin D (CD) prevents the continuous addition of actin monomers to the barbed end of F-actin, leading in time to the persistence of short 'disrupted' filaments (84). The Red Sea sponge product latrunculin B (LB) prevents the continuous formation of actin filaments by complexing with and scavenging actin monomers (374). The Indio-Pacific sponge peptide, jasplakinolide, stabilizes actin filaments by binding to both barbed and pointed ends thus preventing their dynamic turnover (50). The marine sponge macrolide swinholide-A prevents actin subunits from participating in actin-filament nucleation or elongation reactions, typically branching (51), thereby preventing stimulus-induced cortical actin remodelling, without affecting the intact actin network. These compounds have been useful tools to test the requirement for polymerized actin in various endpoints in intact cells. A
summary of the mechanism of action of CD. LB. swinholide-A and jasplakinolide is shown in Figure 1.9.

Figure 1.9 Site of action of actin disrupting agents used in this study

Currently, the mostly widely-used procedure for visualizing the actin cytoskeleton in fixed cells involves the use of fluorescently-labeled phalloidin (Figure 1.8). Phalloidin is a mushroom-derived compound that exclusively binds F-actin (93), making fluorescent conjugates of phalloidin ideal probes to visualize stimulus-dependent changes in actin polymerization. In addition to fluorescent phalloidin probes, monoclonal actin antibodies have proven useful for indirect immunofluorescent detection of different actin isoforms, although these antibodies do not discriminate between G-actin and F-actin forms (136, 421).
Participation of the Actin Cytoskeleton in Insulin-Regulated GLUT4 Traffic

As described above, the actin network of mammalian cells consists of very dynamic structures which continuously form, disassemble and reorganize, and which participate in many cellular processes. One process relevant to studies on insulin action is the actin-based compartmentalization of intracellular organelles. Many intracellular vesicle movements are driven along actin filaments by motor proteins termed unconventional (non-muscle) myosins that convert the chemical energy of ATP hydrolysis directly into movement (31). There is strong evidence of the involvement of different unconventional myosins in mediating various intracellular vesicular traffic events in diverse cell types (274).

As with many other intracellular organelles, considerable evidence points to the potential participation of the actin network in the insulin-dependent traffic of GLUT4-containing organelles [reviewed in (420)]. Inhibition of actin filament polymerization with CD or LB completely blocks insulin-dependent translocation of GLUT1, GLUT3 and GLUT4, and the stimulation of glucose transport in L6 muscle cells (415. 451). In 3T3-L1 adipocytes, CD reduces insulin-dependent GLUT1 translocation by 75% and CD and LB inhibit GLUT4 translocation by 40-50% (453. 317. 112. 42), and this mirrors CD’s effect of on insulin-stimulated glucose transport. i.e. a 40% reduction (453). Finally, actin depolymerization also inhibits GLUT4 translocation and glucose uptake in rat adipocytes (314). None of these treatments impair proximal events in the insulin signal transduction cascade, suggesting that an intact actin network is required for the relocalization of GLUT4 from intracellular storage sites to the cell surface. The exact role of the actin cytoskeleton in the insertion of GLUT4 at the plasma membrane is not known, and is the basis for the study presented in Chapter 3.

It is well recognized that, concomitant with the stimulation of glucose transporter translocation, insulin causes a rapid and marked reorganization of actin filaments below the plasma membrane, promoting membrane ruffling in insulin-responsive cells such as myotubes.
The remodelling of actin filaments in response to insulin is dependent on PI3-K activation (415, 453). In other cell types, insulin has been shown to increase actin stress fiber formation (342, 217). Whether the reorganization of the subplasmalemmal actin network caused by insulin is necessary to allow exocytic GLUT4 vesicles to fuse with the plasma membrane is not known as previous studies with CD and LB lead to disruption of both existing and reorganized actin filaments. To date any connection between actin remodelling and GLUT4 translocation remains correlative because preventing membrane ruffle formation by strategic interference with insulin signalling also diminishes GLUT4 translocation (78, 447, 446). The functional consequence of insulin-dependent actin reorganization is explored in the studies presented in Chapter 3.

In contrast to insulin, the stimulation of GLUT translocation by DNP (i.e. the metabolic demand pathway) does not require an intact actin cytoskeletal network (418), nor does DNP cause actin remodelling (418). Therefore the actin cytoskeleton may represent another potential distinguishing feature of the insulin-dependent glucose transport pathway which does not participate in the glucose transport response to metabolic demand.

**The Microtubule Network**

The tubulin network of cells is a very important and dynamic cytoskeletal structure that is involved in several cellular functions including mitosis, directed motility, intracellular transport, maintenance of cell shape and polarity, and movement of intracellular organelles (240). The microtubule (MT) network is composed of hollow tubes formed by tubulin dimers. Two main isoforms of tubulin exist in mammalian cells, α and β tubulin. which are globular proteins of about 450 amino acids (3) that form a functional αβ dimer. Elongated tubulin protofilaments form by the linear interaction of the β subunit of one dimer with the α subunit of the next. Thirteen tubulin protofilaments are arranged side by side around an empty central core resulting
in the formation of a tubule-like structure with a diameter of 25 nm. The tubulin protofilaments are aligned in parallel along the MT, giving the structure polarity which, like actin filaments, has a fast growing plus end, and a slow growing minus end. MTs emerge from a specialized juxtanuclear structure termed the MT organizing centre (MTOC), from which they spread throughout the cell towards the plasma membrane (258). The plus end of MTs is thought to be located at the plasma membrane where MTs polymerize and depolymerize rapidly.

Involvement of Microtubules in Insulin Action

The evidence for microtubule (MT)-based intracellular traffic of certain organelles is very strong. It is well-known that MTs are required to maintain the organization of both the endoplasmic reticulum (ER) and the Golgi complex by positioning and moving vesicles containing newly synthesized proteins from the ER towards the Golgi along MT tracks (229). Two classes of mechanoenzymes which serve as motors for vesicle movement along MT rails are dyneins and kinesins. Both proteins are cytoplasmic and possess microtubule-activated ATPase activity which allows them to slide along MTs. Dyneins characteristically move vesicles and organelles along MTs in a plus to minus “retrograde” direction, whereas kinesins function in a minus to plus “anterograde” fashion (428).

The involvement of MTs in the traffic of different types of organelles has lead to the examination of their role in GLUT4 traffic in insulin-responsive cells. Disrupting MT structures with pharmacological agents (nocodazole, colchicine or vinblastine), or interfering with MT motor protein function (see below), reduces insulin-stimulated glucose uptake in 3T3-L1 adipocytes by 40%-100%, depending on the study (117, 313, 143, 317). These strategies of interfering with tubulin filaments do not affect insulin signalling (IRS-1 phosphorylation, PI3-K activation) (313, 117), suggesting that MTs are required for the later stages of insulin action, e.g. GLUT4 vesicle integrity or mobilization. (Nonetheless, some studies have pointed out non-
specific effects of these drugs on IRS-1 or Akt phosphorylation [(117) and Patel and Klip, unpublished observations]. Indeed, disruption of the MT network in 3T3-L1 adipocytes causes a marked dispersal of the normal perinuclear intracellular GLUT4 distribution (143). Thus depolymerization of MTs may prevent the biogenesis and proper sorting of pre-existing and newly-synthesized GLUT4 due to disruption of the Golgi network (117), making the "insulin-sensitive" GLUT4 pool unavailable for recruitment by the hormone. Alternatively, three lines of evidence suggest that MT tracks are required for motor-driven movement of GLUT4 organelles to the plasma membrane of 3T3-L1 adipocytes: 1) The linear movement of EGFP-GLUT4 to the plasma membrane is lost upon MT disruption (117, 317); 2) Overexpression of proteins that interfere with the motor protein kinesin, or a crude approach of inhibiting dynein motor function (cytoplasmic acidification) prevent GLUT4 arrival at the cell surface (112, 143); 3) α-tubulin was shown to copurify with GLUT4 compartments (143), and IRS-1 and GLUT4 endomembranes associate with MTs in vitro (313). Therefore, the exact role of the MT network in insulin-stimulated GLUT4 traffic still requires extensive investigation.

**The Intermediate Filament Network**

Intermediate filaments (IFs) are so-named because of their 10-nm diameter which is intermediate between that of actin microfilaments (6 nm) and microtubules (25 nm). In contrast to the evolutionarily conserved actin and MT networks, IFs display diversity in their numbers, sequences, and abundance (127), consistent with the notion that the IF cytoskeleton is tailored to suit specific structural needs of different eukaryotic cells. Despite their diversity, IF superfamily components share a common structure consisting of a dimer made of two α-helical chains oriented in parallel and intertwined in a coiled-coil rod. The antiparallel linear association of 4 rods produces protofibrils; 3-4 protofibrils intertwine to produce an apolar 10 nm-diameter IF (315).
Although the physiological function of IFs is not entirely understood, they are thought to structure the cytoplasm and to resist stresses externally applied to the cell (128). IFs are highly abundant in keratinocytes and neurons where they serve as important structural scaffolds (128). Many other studies have revealed specific contributions of different IF proteins in several fundamental processes such as vimentin in sphingolipid biosynthesis (135) and keratin filaments in protecting hepatocytes from apoptotic stress (61). The participation of IF in intracellular organelle transport has not been examined in detail.

**Participation of Intermediate Filaments in Insulin Action**

IFs are much less dynamic than actin or tubulin filaments but can tether to both cytoskeletal structures. The lack of drugs to experimentally destabilize IFs has limited the analysis of the participation of IFs in cellular events. However, recent studies point to their potential involvement in GLUT4 traffic: Immuno-purified GLUT4-containing compartments isolated from 3T3-L1 adipocytes co-sediment with the IF protein vimentin (143). Furthermore, microinjection of a vimentin-derived peptide into 3T3-L1 adipocytes causes dispersal of the GLUT4 compartment (143), reminiscent of the effect of MT disruption. Finally, IRS-1 and purified GLUT4 compartments contain a poorly defined filamentous component(s) (72) which resembles structures of the IF cytoskeleton. Given similar observations of an association of GLUT4 and IRS-1 proteins with MT filaments (313), the interplay between IF and the other cytoskeletal networks makes IF filaments likely candidates to also participate in insulin-regulated GLUT4 traffic events. Future studies will be required to test this possibility.

**SIGNALLING PATHWAY REGULATING THE ACTIVATION OF GLUCOSE TRANSPORTERS BY INSULIN AND ENERGY DEMAND**

The recruitment hypothesis predicts that there would be no need for the activation of transporters by insulin or exercise, but instead that the rise in glucose transport could be exclusively explained by a gain in glucose transporter number at the surface of muscle cells.
However, as in the case of their cognate tissues, for both L6 myotubes and 3T3-L1 adipocytes, the increase in GLUT4 in isolated membranes is less than the full extent of stimulation of glucose uptake. The possibility is now gaining acceptance that insulin may confer the less active translocated transporters with a higher ability to transport glucose, either by increasing their intrinsic activity or turnover number. This hypothesis is supported by several observations: 1) Although overexpression of the catalytic subunit of PI 3-kinase significantly increases GLUT4 translocation and, in some studies, glucose transport, the stimulation is submaximal at best (125); 2) Treatment of intact 3T3-L1 adipocytes with cell permeable PI-3,4,5-P3, (the PI3-K product) rescues the inhibition of insulin-stimulated glucose transport by wortmannin, but alone does not elevate glucose uptake (174), although it causes GLUT4 translocation (395). These results suggest that activation of PI3-K alone is not sufficient to fully stimulate glucose transport and that insulin may activate another parallel pathway that synergizes with PI 3-kinase to fully stimulate the glucose transport.

Insight into this parallel pathway evolved from studies using the compound SB203580, a potent inhibitor of p38 mitogen-activated protein kinase (p38 MAPK). Treatment of rat skeletal muscle, L6 myotubes or 3T3-L1 adipocytes with SB203580 diminishes insulin-stimulated glucose uptake despite normal insulin-dependent translocation of GLUT1 and GLUT4 to the cell surface (394, 373). These results are interpreted to indicate that following the translocation of GLUTs to the cell surface, they must be activated by a p38 MAPK-dependent signalling pathway (or a SB203580-sensitive intermediary).

Like insulin-stimulated glucose uptake, evidence has also accumulated for an activation component of exercise-stimulated glucose uptake. Combination of treadmill exercise with a maximum insulin treatment causes a partially additive effect on muscle glucose uptake, that does not match the increase in plasma membrane GLUT4 (46, 107). p38 MAPK may also
participate in the activation of glucose transporters during contractions because the enzyme is activated during exercise (373). Furthermore, in cells lacking GLUT4, many energy stressors including hypoxia and chemical hypoxia have been reported to stimulate the rate of glucose transport by activation of GLUTs pre-existing in the plasma membrane. For example, a brief exposure of Clone 9 cells (a non-transformed rat liver cell line which only expresses GLUT1) to cyanide or azide results in a 6-10-fold stimulation of glucose transport, with no detectable increase in the content of GLUT1 at the plasma membrane (366). Similar results are reported in erythrocytes which also only express GLUT1 exclusively at the plasma membrane (100, 484). The molecular mechanism underlying the apparent activation of GLUT1 remains to be identified, but it has been proposed that protein-protein interaction between the transporter and putative regulatory protein(s) underlie the response (366). Whether the p38 MAPK pathway contributes to the apparent activation of glucose transporters in the metabolic demand pathway has yet to be explored. This important aspect of glucose transport regulation is not explored in this Thesis.

PATHOLOGICAL CONDITIONS ASSOCIATED WITH IMPAIRED GLUCOSE UTILIZATION

Glucose homeostasis is impaired in patients with Type 2 diabetes and during other pathophysiological states of insulin resistance including obesity and the insulin resistance syndrome (also known as syndrome X) (491, 77). Hyperglycemia, the hallmark of Type 2 diabetes, is a direct consequence of insulin resistance of the muscle, adipocytes, and liver. The reduction in glucose uptake by the diabetic muscle or adipocyte results from defects in either the expression of glucose transporters, or in their translocation to the plasma membrane. Evidence supporting both possibilities has been demonstrated in animal models of insulin resistance or Type 2 diabetes and in Type 2 diabetic humans. For example, total GLUT4 protein in adipocytes is diminished in many rodent models of Type 2 diabetes (145, 215, 416, 133, 224)
and in adipose cells taken from Type 2 diabetic patients (318). Interestingly, despite decreased GLUT4 expression in adipocytes in diabetic animals and humans, skeletal muscle GLUT4 levels remain normal (224, 215, 318), with the exception of a recent report of diminished GLUT4 levels in slow-twitch muscle fibers of Type 2 diabetic patients (132). For the most part, the diminished insulin-mediated glucose uptake observed in muscle of Type 2 diabetes patients and animal models of the disease is associated with lower insulin-induced translocation of glucose transporters to the plasma membrane (202, 490, 180).

The reduced translocation of GLUT4 to the plasma membrane may occur at any of the entry points in the cascade of events triggered by insulin: 1) One or more steps in the insulin signalling pathway may be affected; 2) The intracellular movement of GLUT4 to the plasma membrane may be impaired (i.e. cytoskeleton, vesicle transport machinery); 3) The glucose transporters may not be able to functionally incorporate into the plasma membrane upon arrival (i.e. SNARE protein defects); 4) The activation of glucose transporters at the membrane may be impaired. To date, there is considerable evidence for alterations at the level of signalling (e.g. Level 1) in animal models of Type 2 diabetes. There is a reduction in insulin receptor phosphorylation in skeletal muscle of animal models of diabetes (247), and muscle and adipose cells of patients with Type 2 diabetes (259, 12). Impaired IRS-1 phosphorylation has also been detected in muscle and liver of diabetic animals (345, 349) and muscle cells of diabetic humans (40). The reduced phosphorylation of IRS-1 is linked to a major decrease in PI3-K activity in response to insulin (349, 158, 40). In addition, a natural mutation in PI3-K (p85α gene) has been associated with insulin resistance in human subjects studied (25). Furthermore, insulin-stimulated PI3-K, aPKC, and Akt1 kinase activities are substantially diminished in skeletal muscle and adipocytes of the lean diabetic Goto-Kakizaki (GK) rat (232, 184). Finally, Akt is inhibited to some extent in diabetic rats (309), and in Type 2 diabetic patients (233). Thus.
defects in insulin signalling through IRS-1, PI3-K, Akt, and aPKC may contribute to the reduced plasma membrane GLUT4 content in Type 2 diabetes. It will be important to differentiate which defects are primary and which arise as a consequence of the hyperglycemia, further exacerbating insulin resistance.

Whether defects also exist in events distal to the initial signalling cascade (e.g. GLUT4 exocytosis) has not been examined to date. As more knowledge is generated on how GLUT4 is mobilized, inserted and activated in the plasma membrane, we will be better-equipped to analyze whether either of these phenomena are altered in the disease state.

---

**RATIONALE, HYPOTHESIS AND ORGANIZATION OF THE STUDY**

In most mammalian cells including skeletal muscle and adipose tissues, the transport of glucose across the cell membrane is rate-limiting for its use. In particular, skeletal muscle is the primary site for insulin-regulated glucose utilization in the post-prandial state. The mechanism by which insulin directs the insertion of GLUT4 proteins into the plasma membrane is not fully understood, but may involve the actin-based cytoskeleton. Independently from insulin, glucose transport can also be rapidly stimulated by a variety of physiological conditions that increase energy demand, such as exercise and response to compromised oxidative metabolism. The mediators of the insulin-independent pathway remain unknown, although evidence supports a role for Ca²⁺, cPKC, and AMPK as mediators of this pathway. The major defect in Type 2 diabetes is insulin resistance of glucose utilization (specifically, defective translocation of glucose transporters) in muscle tissue; yet the ability of contractions to stimulate the muscle to take up glucose is not impaired in Type 2 diabetes. It is therefore important to understand the diverse means to control glucose uptake by insulin and by metabolic demand. To fully understand events involved in GLUT traffic, biological methods at the cellular level need to be
engaged, and therefore muscle cell tissue cultures provide an ideal system with flexibility in cell treatment methods and reasonable homogeneity of responses. Understanding the cellular mechanisms regulating glucose uptake is pivotal for the design of better approaches to improve insulin action in Type 2 diabetes. Therefore, the clinical potential of the proposed studies is that

1) **Actin remodelling may be a useful therapeutic target for improving the delivery of GLUT4 to the cell surface during insulin resistance:** and 2) **some of the insulin-independent pathways may be modulated by agents other than insulin, to improve glucose utilization in the face of insulin resistance of glucose uptake in the muscle.**

**CHAPTER 3: ROLE OF THE ACTIN NETWORK IN INSULIN ACTION**

The preceding Background section discussed the evidence that an intact actin network is required for insulin-dependent GLUT4 mobilization to the plasma membrane of L6 myotubes, 3T3-L1 adipocytes, and rat adipocytes. Insulin causes a rapid and dynamic remodelling of cortical actin filaments: however there has been limited analysis of the morphology and functional consequences of these changes in actin. Growing evidence suggests that the actin-based cytoskeleton plays a role in maintaining the fidelity of signal transmission by compartmentalizing signalling molecules and organelles. The link between the actin microfilament network, the location of insulin signalling molecules, and the location of GLUT4 organelles remains poorly understood. *The studies presented in Chapter 3 test the hypothesis that the actin cytoskeleton facilitates delivery of insulin signals to the insulin-responsive GLUT4 compartment and participates in the recruitment of GLUT4 to the plasma membrane.*

**CHAPTER 4: MEDIATORS OF ACUTE GLUCOSE TRANSPORT REGULATION IN RESPONSE TO METABOLIC DEMAND**

As discussed in the Background section, the mitochondrial uncoupler DNP mimics some effects of exercise or hypoxia *in vivo.* In addition to being PI3-K-independent, other key differences from insulin exist in the exercise/hypoxia-stimulated pathway leading to muscle
glucose influx. Particularly, exercise-induced glucose transport utilizes a change in cytosolic Ca$^{2+}$ whereas stimulation by insulin occurs independent of changes in cytosolic Ca$^{2+}$. Given the many roles of Ca$^{2+}$ as a secondary messenger in a variety of cellular processes, the possibility that the cation mediates the alternative pathway of glucose transport is intriguing. Moreover, it has been reported that phorbol esters that activate conventional, Ca$^{2+}$-sensitive protein kinase C (cPKC) can stimulate glucose uptake in L6 muscle cells. but that the response to insulin of glucose transport does not involve cPKC. Hence cPKC may participate in an the metabolic demand glucose transport pathway. Finally, recent studies have implicated the fuel sensing enzyme AMPK in contraction-dependent GLUT4 translocation in skeletal muscle. Chapter 4 tests the hypothesis that cytosolic Ca$^{2+}$ and its PKC effectors, as well as AMPK may participate in the response of glucose transport to interference with the oxidative chain by the hypoxia-mimetic agent DNP in L6 muscle cells.

CHAPTER 5: MECHANISMS OF CHRONIC GLUCOSE TRANSPORT REGULATION IN RESPONSE TO METABOLIC DEMAND

In addition to the acute increase in glucose uptake induced by energy stressors and insulin, a further increase in glucose uptake beyond the acute phase maximum occurs during chronic exposure to these stimuli. This increase is known to result from an elevated cell content of GLUT1 and GLUT3 proteins, but not GLUT4 (221, 398). Prior studies have shown that, unlike insulin, the rise in glucose transport during prolonged DNP treatment is insensitive to the protein synthesis inhibitor cycloheximide, however the transporter(s) responsible for sustaining glucose transport was not investigated. Given that DNP does not trigger any of the mitogenic signals of insulin in mediating increased GLUT expression (398), it is of interest to elucidate the effects of DNP at the molecular level on the expression of these transporters. Therefore, in Chapter 5, the effect of chronic (18 h) treatment with DNP on GLUT1 and GLUT3 protein and mRNA levels is analyzed and compared to the response to prolonged insulin treatment. It is
hypothesized that, as in the acute response to DNP, prolonged exposure to DNP also exerts a different cellular response from insulin to elevate glucose transport: whereas insulin increases GLUT1 and GLUT3 expression by elevating their biosynthesis, it is hypothesized that DNP elevates GLUT content by preventing its degradation.
Some of the background presented in this chapter has appeared in the following publication:

THE L6 SKELETAL MUSCLE CELL LINE AS A MODEL OF SKELETAL MUSCLE

Although methods to study the regulation of glucose transport in isolated skeletal muscle and adipose tissue are continuously being developed, there is a need for more amenable models to study glucose transport, such as tissue cultures. Tissue culture cell monolayers have the advantages of being homogeneous and offer even exposure to exogenously added reagents. Moreover, they are amenable to transfection by diverse cDNAs. Two cell lines of insulin-responsive tissues have been widely used for understanding glucose transport systems — the rat L6 skeletal muscle line and the mouse 3T3-L1 pre-adipocyte cell line. These cell lines constitute the only reliable source of GLUT4 glucose transporters ex vivo, and ample documentation is available on their ability to reenact the recruitment of glucose transporters, particularly GLUT4 but also GLUT1, in response to insulin. Both cells can reasonably reproduce the quantitative response of their corresponding mature tissues. L6 myotubes respond to insulin with a rapid doubling in the rate of glucose uptake (compared to 2-7 times observed in specific adult muscle fibres). Similarly, 3T3-L1 adipocytes respond by increasing glucose uptake 6-12 times, compared to up to 10 times in rat adipocytes. Notwithstanding these quantitative differences, there is wide documentation of the translocation of GLUT4 transporters to the plasma membrane in both L6 myotubes and 3T3-L1 adipocytes, supported by studies employing diverse techniques ranging from subcellular fractionation and analysis of glucose transporter number, glucose transport activity, photolabelling of cell surface glucose transporters, immunolocalization in intact and permeabilized cells, and immunogold localization in ultrathin sections of the same.

The L6 cell line was derived from chemical transformation of neonatal rat thigh skeletal muscle satellite cells and retains many morphological, biochemical and metabolic properties of skeletal muscle (469), making it a useful in vitro system to characterize physiological responses in muscle such as glucose transport. When cultured in low serum concentrations, L6 cells
differentiate spontaneously after confluency from single cell myoblasts into elongated multinucleated myotubes (209), mimicking the fusion of mononucleated myoblasts during fetal and postnatal life, and during muscle regeneration. Figure 2.1 illustrates the morphology of the L6 cell line in the myoblast and myotube stages.

Figure 2.1 The morphology of L6 myoblasts and myotubes

L6 myoblasts (left panel) and myotubes (right panel) were grown on glass coverslips for 2 and 7 days, respectively, and were fixed in 2.5% (vol/vol) gluteraldehyde and 50% ethanol. The nuclei were stained with Giemsa solution and were viewed by phase contrast microscopy. (The images shown were provided by Atsunori Ueyama).

During the fusion and differentiation process, different sets of genes are either expressed or turned off in a tightly regulated fashion (358) including myogenin, a muscle-specific activating factor which controls the expression of many muscle specific genes during the myogenic process (467). Other muscle-specific proteins upregulated in L6 myotubes include myosin heavy chain, acetylcholine receptor, and creatine kinase (39, 278). GLUT4 protein expression also occurs after differentiation from myoblasts into myotubes (279) resulting in the expression of three glucose transporter isoforms in myotubes: GLUT1, GLUT3 (35) and GLUT4. In the myotube stage, L6 cells contain a large cytoplasmic space rich in actin filaments and are highly amenable to morphological analysis at the light microscopic level. Though α-actin is expressed in myotubes, it is not reorganized into sarcomeres and myotubes do not display cross-striations.
This is in contrast to the cytoplasm in both muscle and fat tissue which is compressed by either myofibrils or the liquid fat droplet, making intracellular compartments poorly resolved by morphological approaches.

For many studies presented in this Thesis, the clonal L6 line used was stably transfected with GLUT4 containing a 14 amino acid myc sequence at its first extracellular loop (see method below). Introduction of a c-myc epitope tag into GLUT4 allows for the direct detection of the presence of GLUT4 at the cell surface of intact cells and for following its journey within the cell. This method of detection is more rapid and less laborious than conventionally used assays of GLUT4 translocation such as subcellular fractionation and immunofluorescence approaches. We have reported previously that expression of GLUT4myc in L6 myoblasts leads to the segregation of the protein to a GLUT4-specific pool, conferring insulin sensitivity to glucose uptake (425). This conclusion is based on the finding that in L6-GLUT4myc myoblasts, the intracellular GLUT4myc compartment contains the majority of the insulin-regulatable amino peptidase (IRAP, a marker of GLUT4 vesicles) but less than half of the GLUT1 housekeeping glucose transporter. Furthermore, unlike other fibroblast cell lines stably expressing GLUT4myc (e.g. Chinese Hamster Ovary cells), a portion (approx. 50%) of the intracellular pool of L6 GLUT4myc is already segregated into a specialized compartment separate from the transferrin receptor-positive recycling endosome (V. Randhawa and A. Klip, unpublished observations). Therefore L6 GLUT4myc cells are endowed with a specialized GLUT4-containing compartment, already at the myoblast stage. By expression of GLUT4myc alone the sensitivity of glucose uptake to insulin is markedly improved, compared to parental myoblasts which do not yet express GLUT4 at this undifferentiated stage. In a study where I participated in characterizing this cell line, we found that, under basal conditions, 90% of the GLUT4myc resides intracellularly. Stimulation for 30 min with 100 nM insulin elevates the cell surface
content of GLUT4myc by 2-fold, with a commensurate reduction in intracellular GLUT4myc (451).

**REAGENTS**

Human insulin was a kind gift from Eli Lilly Canada, Inc. (Toronto, ON, Canada). Bovine serum albumin (BSA), cycloheximide, cytochalasin B, cytochalasin D (CD), dinitrophenol (DNP), o-phenylenediamine dihydrochloride (OPD) reagent, phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin A, E-64, sodium pyrophosphate, NaF, benzamidine, Na,VO₄, phorbol ester (PMA), rotenone, CoCl₂, and wortmannin were obtained from Sigma Chemical (St. Louis, MO, USA). Okadaic acid was from Biomol (Plymouth Meeting, PA, USA). Jasplakinolide, swinholide-A, ProLong Antifade mounting solution, FITC-conjugated concanavalin-A, rhodamine-phalloidin, and Oregon Green-phalloidin were purchased from Molecular Probes (Eugene, OR, USA). Bisindolylmaleimide-I (BIM-I), 1,2-bis(o-Aminophenoxy)ethane-N,N',N''-tetraacetic acid tetra (acetoxyethyl) ester (BAPTA/AM), lactacystin, E-64d, and latrunculin B (LB) were from Calbiochem (La Jolla, CA, USA). The PKC-β inhibitor LY379196 was a kind gift from Eli Lilly and Company (Indianapolis, IN, USA). Octaethylene glycol dodecyl ether (C₈E₈) was purchased from Fluka (Ronkonkoma, NY, USA). Purified L-α-phosphatidylinositol (PI) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Bicinchoninic acid (BCA) protein assay reagent was purchased from Pierce (Rockford, IL, USA). SDS-PAGE and immunoblotting apparatus, polyvinylidene difluoride membranes, dithiothreitol, and Bradford protein assay reagent were from Bio-Rad Laboratories (Hercules, CA, USA). Oxalate-treated TLC Silica gel H plates (250 microns) were purchased from Analtech (Newark, DE, USA). Protein A- and Protein G-Sepharose was from Pharmacia Biotechnology Inc. (Uppsala, Sweden). [³⁵S]methionine, [³²P]dCTP, [³²P]ATP, Amplify, and enhanced chemiluminescence reagents were purchased.
from Amersham Inc. (Oakville, ON, Canada). [3H]-2-deoxyglucose was obtained from DuPont (Boston, MA. USA). Crossstide peptide, and protein kinase A and C inhibitor peptides were obtained from Santa Cruz (Santa Cruz, CA. USA). PKC kinase assay kit and [35S]Ser-PKCe(AA153-164)-NH₂ substrate were purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Paraformaldehyde was purchased from Canenco Inc. (St. Laurent, PQ. Canada).

ANTIBODIES
Polyclonal antibody to PKC-α, PKC-βII, and PKC-γ used for immunoblotting were purchased from Signal Transduction Laboratories (Lexington, KY. USA). Polyclonal antibodies against the 110 kDa subunits of PI3-K (p110α and p110β), atypical PKCs (PKCζ, PKCλ, and PKC-λζ), Akt (Akt1 C260), and p70 S6 kinase were obtained from Santa Cruz (Santa Cruz, CA, USA). Polyclonal antibodies against phosphorylated Akt (serine 473 and threonine 308) were purchased from New England Biolabs (Beverly, MA. USA). Monoclonal anti-phosphotyrosine antibody was purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Monoclonal antibodies to actin (recognizing all isoforms) and to α-actin and β-actin were supplied by Sigma-Aldrich (Oakville, ON, Canada). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Amersham Inc. (Oakville, ON, Canada). FITC-conjugated goat anti-mouse and anti-rabbit, and Cy3-conjugated goat anti-mouse and anti-rabbit antibodies, and 30-nm gold-conjugated goat anti-mouse antibodies were purchased from Jackson Immunoresearch (Westgrove, PA. USA). [125I]Protein A and [125I]sheep anti-mouse IgG were purchased from ICN (Irvine, CA, USA). Polyclonal antibody to GLUT4 used for immunoblotting was purchased from Genzyme (Cambridge, MA, USA). Polyclonal antibody to the insulin receptor α-subunit, and monoclonal antibody against myc (9E10) were obtained from Santa Cruz (Santa Cruz, CA, USA). Polyclonal antibodies to VAMP2, VAMP3, syntaxin-
and SNAP-23 were generated as described previously (444, 466). Polyclonal anti-GLUT3 antibody was generated as described previously (431). Polyclonal antibody against GLUT1 used for immunoprecipitation was generated as previously described (351). Polyclonal antibody against the p85α regulatory subunit of PI3-K. and monoclonal antibody against IRAP (vp165) were kindly provided by Dr. Morris Birnbaum (University of Pennsylvania, Philadelphia, USA). Polyclonal antibody to IRS1 was kindly provided by Dr. Morris White (Joslin Diabetes Centre, Boston, USA). Monoclonal antibody against cPKC used for immunoprecipitation and polyclonal antibody to PKCβI used for immunoblotting were kind gifts from Kinetek Pharmaceuticals, Inc. (Vancouver, Canada). Polyclonal anti-GLUT1 antibody used for immunoblotting was a kind gift from Dr. R. Reithmeier (University of Toronto, Canada). Monoclonal antibody McKI to the α1-subunit of the Na⁺-K⁺-ATPase was a kind gift from Dr. K. Sweadner (Massachusetts General Hospital, Boston, USA). Polyclonal antibody directed against the α2 subunit of AMPK was a gift from Dr. Neil Ruderman (Boston University Medical Center, USA).

CELL CULTURE AND TRANSFECTIONS

Cell Lines and Cell Culture

Tissue culture medium, serum, and other tissue culture reagents were obtained from Life Technologies (Burlington, ON, Canada) or Wisent Inc. (Montreal, PQ, Canada). High fusion L6 muscle cells expressing c-myc epitope-tagged GLUT4 (GLUT4myc, see above and below) or parental L6 cells were maintained in myoblast monolayer culture in α-MEM containing 10% (vol/vol) FBS and 1% (vol/vol) antibiotic-antimycotic solution (10,000 U/ml penicillin G, 10 mg/ml streptomycin, and 25 mg/ml amphotericin B) in an atmosphere of 5% CO₂ at 37°C (279). Cells were subcultured by trypsinization of subconfluent cultures using 0.25% (wt/vol) trypsin. For differentiation into myotubes, myoblasts were plated in medium containing 2%
(vol/vol) FBS at ~4x10^4 cells/ml, fed fresh medium every 48 hours and used 6-8 days after plating, when myotubes were plentiful. To maintain myoblasts in culture, cells were seeded at a density of ~2x10^5 cells/ml onto 25-mm diameter glass coverslips in 6-well tissue culture plates in 10% (vol/vol) FBS for 48 h prior to experimentation. For all acute manipulations with insulin or other stimuli, cells were deprived of serum in culture medium for 4-4.5 h at 37°C prior to experimentation and processing.

Construction of L6 Cells Expressing c-myc Epitope-tagged GLUT4 (GLUT4myc).

L6 muscle cells expressing c-myc epitope-tagged GLUT4 (GLUT4myc) were constructed as described (182). GLUT4myc cDNA was subcloned into the mammalian expression vector pCXN (pCXN-GLUT4-myc). L6 myoblasts were transfected with pCXN-GLUT4-myc and pSV2-bsp, a blasticidin S deaminase expression plasmid, and selected with blasticidin S hydrochloride (Funakoshi. Tokyo, Japan). The human c-myc epitope (14 amino acids) was introduced into the first ectodomain of GLUT4 and the epitope does not affect GLUT4 activity (182, 451).

Constructs

Plasmid DNA purification columns were purchased from Qiagen (Mississauga, ON. Canada). Mammalian expression vector for Enhanced Green Fluorescent Protein (pEGFP) was purchased from Clontech (Palo Alto, CA, USA). pcDNA3 was purchased from Invitrogen (Carlsbad, CA, USA). Rac1-N17 and Rho-N17 plasmids were a gift from Dr. Gary Bokoch (Scripps Research Institute, La Jolla, USA). Plasmid containing EGFP-tagged PH domain of GRP1 (GFP-PH-GRP1) and PLCδ (GFP-PH-PLCδ) were kind gifts from Dr. Michael Czech (University of Massachusetts, Worcester, USA). Plasmids containing full-length cDNAs for GLUT1 (prGT4-12), GLUT3 (pmGLUT3-6) and GAPDH were kindly provided by Dr. M.
Birnbaum (University of Pennsylvania, Philadelphia, USA), Dr. C.F. Burant (University of Chicago, USA) and Dr. H. Elsholtz (University of Toronto, Canada), respectively.

**Transfections**

Effectene transfection kits were purchased from Qiagen (Mississauga, ON, Canada). Transfections of myoblasts were performed according to the Effectene product manual, with 3.2 μL of the Effectene reagent used per μg of transfected cDNA. DNA was introduced to the cells for 5 h, and the cells were washed twice with PBS and maintained in culture medium for another 16 h until further experimentation. Cells were deprived of serum in culture medium for 4-4.5 h at 37°C prior to processing for immunofluorescence.

Transfection of L6 myotubes was performed as described above for myoblasts in 12-well plates, with the following modifications: DNA was introduced to the cells at the start of day 4 post-seeding and cells were maintained for another 72 h until experimentation. For single-cell analysis of actin rearrangements or GLUT4myc translocation, 1.2 μg of Rac1-N17 cDNA or Rho-N17 cDNA was cotransfected with 0.3 μg of pEGFP into L6 GLUT4myc myotubes grown on (18 mm diameter) coverslips.

**2-[3H]-DEOXYGLUCOSE AND 3-O-[METHYL-3H]-METHYGLUCOSE UPTAKE ASSAY**

Differentiated L6 myotube monolayers grown in 12-well plates were rinsed with HEPES-buffered saline (HBS: 140 mM NaCl, 20 mM HEPES-Na, 2.5 mM MgSO₄, 1 mM CaCl₂, 5 mM KCl, pH 7.4). Glucose uptake was quantitated by exposing the cells to 10 μM [³H]-2-deoxyglucose (1 μCi/ml) in HBS for 5 min (208). All GLUTs are sensitive to inhibition by submicromolar concentrations of the mold metabolite cytochalasin B, which has become a benchmark of their activity. Therefore, non-specific uptake was determined by quantitating cell-associated radioactivity in the presence of 10 μM cytochalasin B, which blocks transporter-mediated uptake. At the end of the 5 min period, the uptake buffer was aspirated rapidly and the
cells were washed three times with ice-cold isotonic saline (0.9% wt/vol NaCl) to stop all further transport. The cells were lysed for 10 min in 0.05 N NaOH and the associated radioactivity was determined by liquid scintillation counting. Each condition was assayed in triplicate.

Measurements of 3-O-[3H]-methylglucose uptake were carried out as previously described (221). L6 myotubes grown in 6-well tissue culture plates were rinsed in HBS as described above and glucose uptake was quantitated by exposing the cells to 10 μM 3-O-[methyl-3H]-methylglucose (2 μCi/ml) for 2 min. Cells were washed and processed as described for 2-deoxyglucose uptake above, except that 1 mM HgCl₂ was added to the ice-cold isotonic saline solution during washes.

DENSITOMETRIC ASSAY OF SURFACE GLUT4MYC
The myc epitope on GLUT4, exposed to the extracellular milieu, allows for tagging of the epitope in intact cells with enzyme-conjugated anti-myc antibody to quantitatively score the effects of various stimuli, inhibitors and mutants on GLUT4 translocation. The protocol for the cell surface detection of GLUT4myc was developed and published early on in the course of the studies presented in this Thesis (451). All steps were performed at 4°C to prevent significant endocytosis of GLUT4myc from the cell surface. After incubations with or without stimulus (i.e. insulin, DNP), L6 GLUT4myc myotubes grown in 24-well tissue culture plates were washed twice with PBS, then mildly fixed in 3% (vol/vol) paraformaldehyde (PFA) in PBS containing 1 mM Ca²⁺ and 1 mM Mg²⁺ (to prevent cells from lifting) for 3 min at 4°C. After this period, cells were washed three times with PBS, and the residual PFA was neutralized with 0.1 N glycine in PBS for 10 min at 4°C. Cells were then blocked in 3% (wt/vol) bovine serum albumin (BSA) for 10 min, and then reacted with the anti-myc antibody (1:150 dilution) in HEPES-buffered RPMI containing 10% (vol/vol) goat serum containing for 1 h at 4°C. After
four washes with PBS, cells were incubated with horseradish peroxidase (HRP)-conjugated donkey anti-mouse IgG for 30 min at 4°C. The cells were washed four times with PBS, and then fixed again with 3% PFA for 30 min (5 min at 4°C, then 25 min at room temperature), followed by neutralization with 0.1 M glycine in PBS for 10 min at room temperature. After two washes in PBS, 1 ml/well of 0.4 mg/ml o-phenylenediamine dihydrochloride (OPD) reagent (prepared according to the manufacturer’s instructions) was added for 30 min in the dark at room temperature. OPD provides H₂O₂ substrate for catalysis by HRP. The reaction was stopped by the addition of 0.25 ml of 3 N HCl, the supernatant was collected, and the optical absorbance was measured at 492 nm. Each condition was assayed in quadruplicate. Background absorbance was determined by performing the above protocol in 3 wells in the absence of primary antibody, and the average of this value was subtracted from all other readings.

MICROSCOPY

Surface Scanning Electron Microscopy and Backscatter Detection

L6 GLUT4myc cells were grown to the stage of myotubes on (25 mm diameter) glass coverslips placed in six-well plates. Myotubes were deprived of serum for 4-4.5 h and treated with insulin or other agents at 37°C. After these incubations, specimens were prepared for scanning electron microscopy by fixation with 2.5% (vol/vol) glutaraldehyde in 0.1 M PBS for 2 h. Cells were then washed with PBS and fixed a second time in 1% (wt/vol) OsO₄ in PBS for 1 h at room temperature. The cells were dehydrated in graded ethanol and were subjected to critical point drying. Specimens were coated with gold and examined on a JEOL JSM 820 scanning electron microscope.

Backscatter electron microscopy combines scanning electron microscopy with immunogold labelling of surface GLUT4myc. For these experiments, L6-GLUT4myc
myotubes were fixed with 2% (vol/vol) PFA and 0.2% (vol/vol) gluteraldehyde in PBS for 30 min at room temperature prior to immunolabelling with primary anti-myc antibody and secondary goat anti-mouse antibody linked to 30 nm-diameter gold particles. The cells were dehydrated in graded ethanol and were critically dried. Specimens were coated with carbon and examined on a JEOL JSM 820 scanning electron microscope. Composite signals of the scanning mode and the backscattered electrons from the gold particles were acquired simultaneously on photographic film. Images were analyzed using Image J software, a public domain Java image-processing program inspired by NIH Image software.

**Confocal Laser Scanning and Fluorescence Microscopy**

L6 GLUT4myc muscle cells were grown to the stage of myotubes on (25-mm diameter) glass coverslips placed in six-well plates. Myotubes were deprived of serum for 4-4.5 h and treated with insulin or other agents at 37°C. After these incubations, myotubes were fixed with 3% (vol/vol) PFA in PBS for 5 min at 4°C, followed by 20 min at room temperature, then washed with 0.1 M glycine in PBS for 10 min. Cells were then permeabilized with 0.1% (vol/vol) Triton X-100 in PBS for 3 min and then washed with PBS. For labeling of actin filaments, fixed and permeabilized cells were incubated for 1 h at room temperature with rhodamine- or FITC-labelled phalloidin (0.01U/coverslip). To assess autofluorescence, additional samples were treated for 1 h with PBS without labelled phalloidin. For immunocytochemistry, fixed and permeabilized myotubes were first blocked for 10 min in 3% BSA in PBS, then incubated for 1 h at room temperature with various primary antibodies in 3% BSA/PBS. Following this incubation the cells were washed with PBS and subsequently were incubated with either Cy3- or FITC-conjugated goat anti-rabbit or anti-mouse secondary antibodies at a dilution of 1:1000 for 1 h at room temperature. Cell monolayers were washed
with PBS and mounted in ProLong Antifade solution onto glass slides for further examination by fluorescence or confocal laser scanning microscopy.

Cell surface immunostaining of GLUT4myc in non-permeabilized cells was carried in L6 myoblasts or L6 myotubes using a similar protocol as that used for densitometric analysis of surface GLUT4myc (see above) with the following modification: all steps were the same up to addition of the secondary antibody. To detect cell surface GLUT4myc, Cy3-conjugated goat anti-mouse antibody (1:1000) was added for 30 min in the dark at 4°C. Cells were washed and fixed as described above, then were mounted in ProLong Antifade solution onto glass slides for examination by fluorescence or confocal microscopy.

For fluorescence microscopy, cells were examined with an inverted Leica DM-IRB microscope with a 100X/1.3 NA oil immersion objective. Digital images were acquired using a Princeton Instruments Micromax cooled charged coupled device (CCD) camera. For confocal laser scanning microscopy, confocal images were collected on a Leica DM-IRB inverted microscope equipped with a Leica confocal laser scanning imaging system (TCS 4D) or a Zeiss LSM 510 laser scanning confocal microscope. Specimens were studied at a magnification of 100X/1.4 NA oil immersion objective. A series of images was collected at an optical thickness of 0.5 μm along the Z axis sequentially for the red and green fluorescent labels. Confocal images were constructed using NIH Image 1.62 software (Bethesda, MD, USA). For presentation, the contrast and brightness of the fluorescence and confocal microscopy images were digitally enhanced using Adobe Photoshop 4.01 (Mountain View, CA, USA).

MEMBRANE PREPARATIONS

Total Membrane Preparation
After the appropriate treatments, parental L6 myotube monolayers grown on 10-cm diameter dishes were gently scraped in culture media with a rubber policeman, and pelleted by
centrifugation in a table-top centrifuge at 760xg for 10 min at room temperature. Cell pellets were resuspended in 6 ml of ice-cold homogenization buffer (250 mM sucrose, 20 mM HEPES, pH 7.4, 2 mM EGTA, 3 mM NaN₃, containing the following protease inhibitors, freshly added: 200 μM PMSF, 1 μM leupeptin, 1 μM pepstatin A). The samples were then homogenized in a 40 ml Dounce type A homogenizer on ice (20 strokes) and the homogenate was centrifuged at 760xg for 5 min at 4°C. The resultant supernatant was saved on ice: the pellet was resuspended in 3 mL of homogenization buffer and re-homogenized, followed by another 760xg spin for 5 min at 4°C. The pellet was discarded and the supernatant was pooled with the first 6 mL-supernatant, and the total homogenate was centrifuged at 190 000xg in a Ti20 fixed angle rotor for 1 h to sediment the total microsomal (membrane) fraction. The isolated membranes were resuspended in homogenization buffer. Protein content was determined using the BCA assay. Total membrane samples (40 μg) were solubilized in Laemmli sample buffer (239) and subjected to 10% SDS-PAGE and immunoblotting.

**Plasma Membrane-Enriched Fraction**

After specific treatments, parental L6 myotube monolayers grown on 10-cm diameter dishes were gently scraped with a rubber policeman in 5 ml of ice-cold homogenization buffer (as above) and homogenized in a 40 ml Dounce type A homogenizer on ice (20 strokes). The homogenate was centrifuged at 760xg in a table-top centrifuge for 5 min at 4°C, and the resultant supernatant was centrifuged at 31,000xg in a JA20.1 fixed-angle rotor for 20 min to separate a plasma membrane-enriched pellet from an intracellular microsome supernatant. The plasma membrane fraction was resuspended in homogenization buffer. Membrane protein content was determined by the bicinchoninic acid (BCA) method. Fifty micrograms of protein were solubilized in Laemmli sample buffer (239) and separated by 7.5% SDS-PAGE, followed by immunoblot analysis.
Subcellular Fractionation

After specific treatments, L6 GLUT4myc myotube monolayers grown on 15-cm diameter dishes (4 dishes/condition) were gently scraped in culture media with a rubber policeman, and pelleted by centrifugation in a table-top centrifuge at 760xg for 10 min at room temperature. Cell pellets were resuspended in 5 ml of ice-cold homogenization buffer and homogenized by 20 passages through a cell cracker (clearance 0.0016 in) (35). The homogenate was centrifuged at 760xg in a table-top centrifuge for 5 min at 4°C, and the resultant supernatant was centrifuged at 31,000xg in a JA20.1 fixed-angle rotor for 20 min to separate the plasma membrane-enriched fraction (PM, pellet) from the intracellular microsome supernatant. The light density microsomes (LDM) were separated from the high density microsomes (HDM) by centrifugation of the intracellular membrane supernatant at 40,000xg in a SW55 swing-bucket rotor for 20 min at 4°C. The supernatant of this spin was then centrifuged at 190,000xg in a SW55 rotor for 1 h to isolate the LDM fraction (pellet) and cytosolic fraction (CYT, supernatant). Meanwhile, the PM fraction was purified by discontinuous sucrose gradient centrifugation of the crude plasma membrane pellet (above) at 210,000xg in a SW41 swing-bucket rotor for 1 h at 4°C. The LDM and PM pellets were resuspended in 0.5 ml homogenization buffer and their protein content was determined by the BCA method.

Detergent Extraction of Isolated Membranes

For some experiments, subcellular fractions (cytosol, CYT: low density microsomes. LDM; and plasma membranes. PM) were solubilized in homogenization buffer (as above) containing 1% (vol/vol) Triton X-100 and 100 μM DTT by sonication on ice (4 x 5 seconds at 30% capacity) followed by rotary shaking for 1 h at 4 °C. Detergent-insoluble pellets (P) were collected by centrifugation at 100,000xg for 1 h in a TLA 100.3 fixed angle rotor, washed 3 times with homogenization buffer containing 1% Triton X-100, and solubilized in Laemmli
sample buffer (239). The detergent-soluble supernatant (S) was removed. its proteins precipitated with 40% (vol/vol) trichloroacetic acid, then solubilized in Laemmli sample buffer.

**SDS-PAGE AND WESTERN IMMUNOBLOTTING**
Proteins from resultant fractions or cell lysates (see below) were separated by SDS-PAGE (7.5% or 10% polyacrylamide) and transferred electrophoretically onto polyvinylidene difluoride membranes at a constant voltage of 100 V for 2 h at 4°C. Immunodetection was performed as previously described (279). All antibodies were diluted in 150 mM NaCl, 50 mM Tris·HCl pH 7.5, 1% (wt/vol) BSA, 0.04% (wt/vol) Nonidet P (NP)-40, and 0.02% (wt/vol) NaN3. In most experiments, HRP-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies were used to detect bound antibodies, and the complexes were visualized by enhanced chemiluminescence (ECL). Autoradiograms from ECL films were quantified in the linear range by densitometry using NIH Image software. For some experiments, bound antibodies were visualized by [125I]Protein-A for polyclonal antibodies and [125I]sheep anti-mouse for monoclonal antibodies. Detection and quantitation of [125I] was performed with a Molecular Dynamics Storm Phosphorimeter System (Sunnyvale, CA, USA).

**RNA ISOLATION AND NORTHERN BLOT HYBRIDIZATION**
Total RNA from L6 myotubes was extracted by a single-step RNA isolation procedure using guanidium thiocyanate-phenol-chloroform (69). RNA levels were quantified by the 260/280 UV absorbance and electrophoresed under denaturing conditions in 1.2% (wt/vol) agarose gels containing 8% (vol/vol) formaldehyde. RNA was then transferred onto nitrocellulose membranes and baked for 2 h at 80°C in a vacuum oven. Equal loading of all samples containing 20 μg of RNA was confirmed by ethidium bromide staining. The nitrocellulose membranes containing the RNA samples were prehybridized overnight at 42°C with 200 μg/ml salmon sperm DNA in 6X SSPE, 10X Denhardt’s solution, and 0.5% SDS. Hybridization was then performed in 50%
formamide, 6X SSPE, 0.5% SDS, 5% dextran sulphate, and 100 µg/ml salmon sperm DNA for 2 days at 42°C by adding [α\(^{32}\)P]dCTP-labelled GLUT1, GLUT4, or GAPDH cDNAs. Each cDNA was labelled using a random primer DNA labelling kit according to the manufacturer's instructions (Boehringer Mannheim, Belleville, NJ, USA). Following hybridization, nitran membranes were washed 3 times for 5 min using 1X SSC containing 0.1% SDS at room temperature. Membranes were then washed for 30 min with 0.1X SSC containing 0.5% SDS at 42°C prior to exposure to the Molecular Dynamics PhosphorImager System (Sunnyvale, CA, USA) for detection and quantitative analysis of mRNA bands.

[^{35}S]METHIONINE LABELLING AND IMMUNOPRECIPITATION OF GLUCOSE TRANSPORTERS

Labelling of L6 myotubes with \[^{35}S\]methionine and glucose transporter immunoprecipitation was performed essentially according to a previously established procedure (351). L6 myotubes, grown and differentiated in 10 cm plates, were rinsed twice with PBS, pH 7.4 containing 1% (vol/vol) antibiotic-antimycotic solution prior to incubation for 2 h in methionine-free α-MEM supplemented with 2% (vol/vol) dialyzed FBS and 1% (vol/vol) antibiotic-antimycotic. The medium was removed and myotubes were labelled for 4 h in fresh methionine-free α-MEM containing 100 µCi of \[^{35}S\]methionine/plate. The labelling medium was removed and the cells were rinsed twice with PBS. For chasing, complete α-MEM containing appropriate stimuli was added to the labelled cells for 2 h to 48 h, with medium replaced every 12 h to ensure continuous stimulation. Pulsed-and-chased myotubes were washed twice with cold PBS and lysed in 1.0 ml of 2% (vol/vol) C12E6/PBS containing 0.4 mM PMSF, 1 µM leupeptin and 1 µM pepstatin A. The cell lysates were transferred to Eppendorf tubes, vortex-mixed vigorously for 1 min. and centrifuged at 12,000xg in a table-top centrifuge for 15 min at 4°C. A portion of each supernatant was used for determination of protein concentration and trichloroacetic acid-precipitable radioactivity and was stored at -80°C. A
0.9 ml portion of each supernatant was pre-cleared with 10 µl of rabbit pre-immune serum for 1 h at 4°C with gentle mixing, followed by a 2 h incubation with 25 µl of a 50% (vol/vol) suspension of Protein A-Sepharose in PBS. Immunocomplexes were collected by centrifuging the mixture for 1 min at 12,000xg in a microfuge, and the supernatant was used for immunoprecipitation of GLUT3 or GLUT1. Antiserum against either GLUT3 or GLUT1 was added and was incubated for 4 h at 4°C. The immune complexes were collected with Protein A-Sepharose beads as described above and were washed once with 1 ml of Wash I (50 mM HEPES, 1 mM EDTA, 150 mM NaCl and 1% (vol/vol) NP-40), once more with Wash II (Wash I containing 1.0 M NaCl), twice more with Wash I, and finally once with PBS. The immune complexes were incubated in 30 µl 2x Laemmli sample buffer for 30 min at 37°C and were separated by 10% SDS-PAGE. Gels were stained with Coomassie Blue, destained, and soaked for 30 min in Amplify. The polyacrylamide gels were then vacuum dried under heat and suction onto filter paper and exposed to Phosphorimager analysis. Detection and quantitation of [35S]-GLUTs were performed with a Molecular Dynamics Storm Phosphorimager System.

**KINASE ASSAYS**

**Akt Kinase Activity Assay**

Immunoprecipitation of Akt1 and its kinase assay was performed essentially as described previously (218). Cells were lysed with buffer containing 50 mM HEPES, pH 7.6, 150 mM NaCl, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 30 mM sodium pyrophosphate, 10 mM NaF, 1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, 1 mM Na,VO₃, 1 mM dithiothreitol and 100 mM okadaic acid. Anti-Akt1 antibody was pre-coupled to a mixture of Protein A- and Protein G-Sepharose beads by incubating 2 µg of antibody per condition with 20 µl of the Protein A-/Protein G-Sepharose beads (100 mg/ml) for a minimum of 2 h. These anti-Akt1/beads were washed twice with ice-cold PBS and once with ice-cold lysis buffer. Akt1

83
was immunoprecipitated by incubating 200 μg of total cellular protein with the anti-Akt1/beads complex for 2-3 h under constant rotation (4°C). Akt1 immunocomplexes were isolated and washed 4 times with 1 ml wash buffer [25 mM HEPES, pH 7.8, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 0.1% (wt/vol) bovine serum albumin, 1 M NaCl, 1 mM DTT, 1 mM PMSF, 1 μM microcystin, 100 nM okadaic acid] and twice with 1 ml kinase buffer (50 mM Tris/HCl, pH 7.5, 10 mM MgCl2, and 1 mM DTT). The sample was then incubated under constant agitation for 30 min at 30°C with 30 μl of reaction mixture (kinase buffer containing 5 μM ATP, 2 μCi [γ-32P]ATP, 100 μM Crosstide substrate). Following the reaction, 30 μl of the supernatant was transferred onto Whatman p81 filter paper and was washed with 3 ml of 175 mM phosphoric acid 4 times for 10 min. and once with distilled water for 5 min. Filters were air-dried and 32P incorporated into Crosstide was measured by liquid scintillation counting. One unit of protein kinase activity corresponds to 1 μM of 32P incorporated into the substrate peptide under the assay conditions.

For some experiments the activation of Akt was measured by immunoblotting with phospho-specific Akt antibodies. After the appropriate treatments, L6 myotubes grown in 6-well tissue culture plates were lysed on ice in 200 μl of Laemmli sample buffer containing freshly added protease and phosphatase inhibitors (200 μM PMSF, 1 μM leupeptin, 1 μM pepstatin A, 30 mM sodium pyrophosphate, 10 mM NaF, 1 mM benzamidine, 1 mM Na3VO4). The samples were lysed by passage 5 times through a 27-gauge needle and were boiled for 3 min prior to SDS-PAGE and immunoblot analysis.

**PI3-K Activity Assay**

Phosphatidylinositol 3-kinase (PI3-K) activity was assayed in phosphotyrosine immunoprecipitates as described previously (417), with some modifications. L6 myotubes grown in 10 cm-diameter tissue culture plates were washed twice with ice-cold PBS containing
1 mM Na₃VO₄ and lysed in 1 ml of buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 1% (vol/vol) NP-40, 10% (vol/vol) glycerol, 10 mM sodium pyrophosphate, 100 mM NaF, and 1 mM Na₃VO₄, and protease inhibitors (1 μM leupeptin, 1 μM pepstatin A, 10 μM E-64, and 200 μM PMSF). After 15 min of slow agitation, followed by centrifugation (12,000xg, 15 min), the protein concentration of the clarified supernatant was determined by the BCA method. Two hundred μg of cell lysate was subjected to immunoprecipitation using 2 μg of anti-phosphotyrosine antibody for 2 h, followed by 40 μl of a 50% (wt/vol) slurry of Protein A- and G-sepharose beads for 1 h at 4°C. The immunoprecipitates were washed three times with Buffer 1 (PBS containing 1% NP-40, and 100 μM Na₃VO₄), three times with Buffer 2 (100 mM Tris-HCl, pH 7.5, 500 mM LiCl, and 100 μM Na₃VO₄), and twice with Buffer 3 (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 100 μM Na₃VO₄). The pellets were resuspended in 50 μl of Buffer 3 containing 100 mM MgCl₂ and 0.3 mg/ml phosphatidylinositol. The reaction was initiated by the addition of 5 μl of 440 μM ATP containing 10 μCi [γ³²P]ATP. After 10 min at 30°C, the reaction was terminated by the addition of 20 μl of 8 M HCl and 180 μl of CHCl₃-methanol (1:1). The samples were centrifuged for 5 min at 12,000xg, and 50 μl of the lower organic phase was removed and applied to a potassium oxalate (1%) pre-treated Silica gel 60 thin layer chromatography (TLC) plate which had been pre-baked for at least 1 hour at 70°C. Lipids were separated by TLC using CHCl₃/CH₃OH/H₂O/NH₄OH (60:47:11:3) as the running solvent. The detection and quantitation of [³²P]P13-P on TLC plates were performed with a Molecular Dynamics Phosphorimager System.

**p70S6 Kinase Activity Assay**

P70 S6 kinase activity was assayed as described previously (116). Immunoprecipitation of p70 S6 kinase was done using 1 ml of cell lysate prepared as described above for the Akt
assay, and 1 µg of a rabbit polyclonal p70 S6 kinase antibody. The p70 S6 kinase immunocomplex was washed three times with buffer B (50 mM Tris acetate, pH 8, 50 mM NaF, 5 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 mM EDTA, 1 mM EGTA, 10 mM okadaic acid, 0.1% vol/vol β-mercaptoethanol) including all the protease inhibitors used in the Akt assay above. The complexes were then washed twice with buffer C (20 mM 4-morpholinepropanesulfonic acid, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 2 mM EDTA, 20 mM MgCl₂, 2 mM Na₃VO₄ and 1 mM dithiothreitol). P70 S6 kinase activity was assayed in a final volume of 50 µl of buffer C containing 1 µM protein kinase A and C inhibitor peptides, 0.2 mM S6 peptide, and 0.25 mM Mg-[32P]ATP at 30°C for 10 min. Aliquots (30 µl) were transferred onto Whatman p81 filter paper and washed with 3 ml of 175 mM phosphoric acid 4 times for 10 min and once with distilled water for 5 min. ³²P incorporated into the S6 peptide was measured by liquid scintillation counting. One unit of protein kinase activity corresponds to 1 µM of ³²P incorporated into the substrate peptide under the assay conditions.

apKC Kinase Assay

Atypical PKC enzymatic activity was measured in specific immunoprecipitates as described previously (18). Cells were lysed by sonication in buffer containing 20 mM Tris HCl (pH 7.5), 150 mM NaCl, 1.2 mM EGTA, 0.5% (vol/vol) NP-40, 20 mM β-mercaptoethanol, 200 µM PMSF, 1 µM leupeptin, 1 µM pepstatin-A, 1 mM NaF, 1 mM Na₃VO₄. Lysates were first cleared with preimmune rabbit serum, followed by treatment with goat anti-rabbit secondary antibody and 50% (wt/vol) protein-A and -G Sepharose beads for 1 h each at 4°C. apKC was then immunoprecipitated from the resultant supernatants by overnight treatment at 4°C with 5 µl of anti-apKC antibody (recognizing both ζ and λ isoforms), followed by addition of secondary antibody. Precipitates were collected by mixing the immune complexes with 50% (wt/vol)
protein AG-Sepharose beads for 1 h at 4°C. followed by 3 – 1 ml washes with buffer containing 50 mM Tris HCl (pH 7.5), 5 mM MgCl₂, 100 µM Na₃VO₄, 100 µM Na,P₂O₇, 1 mM NaF and 100 µM PMSF. The suspension was then incubated in a thermal mixer for 10 min at 30°C in 100 µl of reaction mixture containing 5 µCi[³²P]ATP, 50 µM ATP, 4 µg phosphatidylserine, and 40 µM [³⁵S]-PKCε(AA153-164)-NH₂ substrate. This PKCε pseudosubstrate peptide is an ideal in vitro aPKC substrate (18). After incubation, an 80 µl aliquot of the reaction mixture was spotted on Whatman p81 filter paper and washed with 3 ml of 175 mM phosphoric acid 4 times for 10 min and once with distilled water for 5 min. ³²P incorporated into the S6 peptide was measured by liquid scintillation counting.

cPKC Kinase Assay

Plasma membranes were resuspended in 0.5 ml of immunoprecipitation buffer (50 mM HEPES, pH 7.8, 1% (vol/vol) Triton X-100, 2.5 mM EDTA, 200 µM PMSF, 1 µM leupeptin, 1 µM pepstatin A, 1 mM benzamidine, 1 mM Na₃VO₄) and were lysed by passing through a 27 gauge syringe 5 times. The homogenate was centrifuged at 12,000xg in a table-top centrifuge for 5 min and the supernatant was incubated overnight with 20 µl of anti-cPKC monoclonal antibody at 4°C with rotary shaking. To this mixture was added 50 µl of 50% (wt/vol) Protein A-Sepharose beads for 1 h. The Sepharose beads and attached proteins were pelleted by centrifugation and washed three times with PBS plus 0.1% (vol/vol) Triton X-100. For immunoblotting, proteins of the pellets were eluted by boiling the samples for 3 min in Laemmli sample buffer. The eluate was subjected to SDS-PAGE and immunoblotted with antibodies to various PKC isoforms. The phosphotransferase activity of PKC in immunoprecipitates from plasma membranes was measured using a PKC assay kit. The assay is based on phosphorylation of a specific substrate peptide (QKRPSQRSKYL) using the transfer of the γ-phosphate of [³²P] ATP by PKC kinase. To the enzyme preparation was added: substrate, lipid
activators, kinase inhibitors and Mg²⁺/ATP reaction buffer (containing 1.5 μCi [³²P]ATP). The mixture was incubated at 30°C for 10 min. The phosphorylated substrate was then separated from the residual [³²P] ATP using Whatman p81 filter paper, washed in 175 mM phosphoric acid, air dried, then quantitated using a liquid scintillation counter.

**AMPK Activity Assay**

AMPK activity was assayed based on the protocol of Vavvas et al. (435). Serum-starved myotubes grown in 10-cm-diameter dishes were treated with appropriate conditions then placed on ice for lysis with 1 ml of buffer containing 30 mM HEPES (pH 7.4), 2.5 mM EGTA; 3 mM EDTA; 70 mM KCl; 0.1% (vol/vol) NP-40 and phosphatase and protease inhibitors (20 mM β-glycerophosphate; 20 mM NaF; 2 mM NaP₂O₇; 1 mM Na₃VO₄; 200 μM PMSF; 1 μM pepstatin A). Lysates were sheared by passage 5 times through a 27-gauge needle, and centrifuged for 10 min at 12,000 x g to remove cell debris and nuclei. Total cellular protein was determined using the BCA protein assay. AMPK was immunopurified with Protein A-G Sepharose beads coupled to antibody directed against the α₂ subunit of AMPK heterotrimer (435). The immunocomplexes were collected washed 3 times with 1 ml buffer containing 50 mM Tris HCl (pH 7.5), 5 mM MgCl₂, 100 μM Na₃VO₄, 100 μM Na₃P₂O₇, 1 mM NaF and 100 μM PMSF. AMPK activity was assayed against 0.44 μg/μl of the SAMS peptide (HMRSAMSGGLHLVKRR) in a final volume of 50 μl of reaction buffer containing 40 mM HEPES (pH 7.4), 80 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, 4 μM ATP, 2 μCi/μl [³²P]ATP at 30°C for 10 min. A 40 μl aliquot of the reaction mixture was spotted onto P-81 Whatman paper. The paper was washed 4 times with 175 mM phosphoric acid, air dried, and associated radioactivity was measured by liquid scintillation counting.
STATISTICAL ANALYSIS

Statistical analysis was performed using the Analysis of Variance (ANOVA) test (Fisher, multiple comparisons). ANOVA allows one to compare the variation between three or more sample means to the variation within the samples themselves. For example, if there are 4 population means, with 5 data values in each sample, then $k$ is the number of populations means based on independent random samples of $n_1$, $n_2$, ..., $n_4$ observations. We want to compare the means for $k=4$ populations based on random samples of $n_1=n_2=n_3=n_4=5$ data points per group. Let $x_{ij}$ represent the $j$th measurement ($j=1, 2, 3, 4, 5$) in the $i$th population group ($i=1, 2, 3, 4$). Then it can be shown that the sum of squares of deviations of all $n_1+n_2+n_3+n_4=20$ values about their overall mean ($\bar{x}$) becomes the total sum of squares (Total SS) as follows:

$$\text{Total SS} = \sum_{i=1}^{k} \sum_{j=1}^{n_i} (x_{ij} - \bar{x})^2$$

Therefore, the total sum of squares of deviations of all observations within all samples results from the sum of two components: the variation (sum of squares) of the sample means, and the variation within the sample themselves (272).
CHAPTER 3: ROLE OF THE CYTOSKELETAL NETWORK IN INSULIN ACTION

The majority of the work presented in this chapter has appeared or will appear in the following publications:


Theodoros Tsakiridis, Evangelina Tsiani, Poli Lekas, Zayna A. Khavat, Gary Sweeney, Romel Somwar, Vera Cherepanov, Ba Tu, Amira Klip, Catherine Whiteside, and Gregory P. Downey. Independent activation of phosphatidylinositol 3-kinase and Ras and their downstream effectors by insulin in L6 muscle cells. (In preparation)
SUMMARY

There has been limited analysis of the insulin-induced changes in actin morphology and the functional consequence of these changes. The actin cytoskeleton can participate in the compartmentalization of intracellular macromolecules and organelles. It is therefore conceivable that insulin-derived signalling intermediaries redistribute in response to actin reorganization. Therefore, the aim of the studies presented in this chapter was to examine the link between changes in the actin microfilament network, the location of insulin signalling molecules, and the location of GLUT4 organelles in L6 myotubes. These cells contain a large cytoplasmic space rich in actin filaments, which is amenable to morphological analysis using microscopic techniques.

The first section of this chapter examines the reorganization of actin microfilaments by insulin at the morphological level. Upon insulin treatment, scanning electron microscopy revealed a dynamic distortion of the dorsal cell surface (membrane ruffles) that resulted from the reorganization of actin filaments to form structures that protruded from the dorsal surface of the myotube. Actin remodelling was prevented by depolymerizing the actin cytoskeleton with cytochalasin D (CD), latrunculin B (LB), jasplakinolide, or swinholide-A, as was insulin-stimulated GLUT4myc externalization. Transient transfection of dominant inhibitory Rac1 (N17), but not N17 Rho, into L6 myotubes prevented formation of dorsal actin structures and blocked insulin-induced GLUT4myc translocation to the cell surface.

The second portion of this chapter examines the participation of insulin-dependent actin filament remodeling in the localization of insulin signalling molecules. In unstimulated cells, immunostaining of the insulin receptor (IR) was evenly distributed throughout the cell surface: IRS-1, p85α, and p110β were diffusely punctate: and p110α appeared to be already tethered to actin stress fibers. Neither the distribution of the IR nor p110β was altered upon stimulation of myotubes with insulin. However, IRS-1 and the p85α and p110α polypeptides colocalized with
the newly formed actin structures caused by insulin stimulation. Given that the PI3-K enzyme was detected in the actin structures, the generation of the PI-3.4.5-P, lipid product in membranes associated with the remodelled filaments was examined by transient transfection of cDNA for a fluorescent ligand of PI-3.4,5-P, GFP-tagged PH domain of GRP1 (GFP-PH-GRP1). Upon stimulation with insulin, GFP-PH-GRP1 was also detected in the insulin-induced actin structures. Furthermore, actin remodelling and GLUT4 externalization were blocked in cells highly overexpressing GFP-PH-GRP1, confirming that PI-3.4.5-P, is required for both phenomena. Akt, (the PI3-K effector which is activated by membrane production of PI-3.4.5-P,.) also appeared in the actin structures, although another PI3-K effector, aPKC, was excluded from the actin filaments. Furthermore, depolymerization of F-actin with CD or LB reduced insulin-dependent Akt and aPKC activation. These results suggest that insulin-dependent actin filament remodelling determines the location of specific intracellular insulin signalling molecules. IRS-1: PI3-K (p85α-p110α). Akt (but not aPKC or the IR), and that actin remodelling spatially coordinates the PI3-K-dependent generation of PI-3.4.5-P, required to activate Akt.

The third portion of Chapter 3 examines the relationship between actin remodelling and the distribution of GLUT4myc in L6 myotubes. GLUT4myc recruitment into the actin-rich structures was observed after 10 min of insulin treatment, although the actin structures were already prominent by 3 min of insulin exposure. IRAP, a transmembrane aminopeptidase that resides in the GLUT4 compartment, morphologically colocalized with GLUT4 vesicles in the actin structures. Fluorescence microscopy and immunogold staining of surface GLUT4myc coupled to backscatter electron microscopy revealed a high density of this protein in areas of membrane ruffles, which was prevented by CD, LB, and jasplakinolide. These findings suggest
that GLUT4 vesicle incorporation into the plasma membrane involves insulin-dependent cortical actin remodelling at defined loci below the cell surface.

In the final section of this chapter, the biochemical association of insulin signalling molecules and glucose transporters with the cytoskeleton was examined by assessing their solubility in the nonionic detergent, Triton X-100. Irrespective of insulin stimulation, the majority of IRS-1, p85, p110α, and p110β were recovered in the Triton X-100-insoluble material that was also enriched with actin, whereas Akt was always soluble. A portion (~50%) of intracellular GLUT4 and GLUT1 sedimented with the Triton X-100-insoluble material. In contrast, IRAP, and the GLUT3 protein were fully soluble in Triton X-100 extracts of both insulin-treated and control myotubes. Furthermore, all plasma membrane-associated GLUT4 and GLUT1 proteins were detergent-soluble. These observations suggest that many insulin signalling molecules, as well as intracellular GLUT4 and GLUT1 are somehow tethered to cytoskeletal structures, which may contain actin.

The findings in this chapter have lead to a comprehensive model for the involvement of the actin microfilament network in the propagation of insulin signal transduction and the redistribution of signalling molecules and glucose transporters.

RESULTS

Insulin-Stimulated Actin Filament Remodelling

Morphological Actin Analysis

Insulin-induced changes in the morphology of the myotube cell surface were first examined using scanning electron microscopy (SEM). For all experiments described herein, a final concentration of 100 nM of human insulin (humulin) was used to maximally stimulate L6 myotubes. Scanning electron micrographs of the L6 myotube monolayer were obtained after 0, 3 min, and 10 min of insulin stimulation (100 nM). In unstimulated myotubes, very little distortion of the dorsal cell surface was observed (Figure 3.1. Basal). At 3 min of insulin stimulation, the plasma membrane showed structures resembling membrane ruffles, particularly
above the nuclei. By 10 min of insulin treatment, the protrusions from the plasma membrane were more pronounced (Figure 3.1). These cell surface protrusions diminished with time, and traces of the membrane ruffles remained after 30 min of exposure to insulin (not shown).

**Figure 3.1 Effect of insulin on L6 myotube surface morphology**

Scanning electron micrographs of the dorsal surface of serum-starved (4 h) L6 myotubes after 0, 3 min, and 10 min of treatment with 100 nM insulin were acquired as described in *Materials and Methods*. The images are representative of 2 experiments. Scale bar: 5 μm. (The results shown in this figure were prepared with the assistance of Dr. Peter Tong.)

Confocal laser scanning fluorescence microscopy was used to compare the distribution of intracellular actin filaments to the membrane ruffles observed in Figure 3.1. Under basal conditions, rhodamine-phalloidin stained long filamentous actin stress fibers that were aligned along the longitudinal axis of the cell (Figure 3.2A). Insulin treatment (100 nM) resulted in a rapid reorganization of actin into structures, reminiscent of those observed by scanning electron microscopy. The new actin-containing structures were apparent within 3 minutes of exposure to the hormone, and proceeded to become more prominent by 10 minutes of insulin stimulation (Figure 3.2A). To determine the approximate height of the new actin structures caused by insulin, Z-scan optical sections were collected at 0.5 μm intervals across the depth of the myotubes. The protrusions were clear through 6-8 optical sections, suggesting that they extended for vertical distances of at least 3-4 μm from the perinuclear region to the dorsal cell surface of the myotube (Figure 3.2B). The horizontal lengths of the actin structures were variable, but tended to extend for up to 40 μm. Towards the ventral surface of the cell, only stress fibers of actin filaments were observed (Figure 3.2B)
Figure 3.2 Insulin induces dorsal, actin-rich structures which extend from the plasma membrane to the perinuclear region

A

<table>
<thead>
<tr>
<th>Basal</th>
<th>3 min</th>
<th>10 min</th>
</tr>
</thead>
</table>

B

| 4 μm above ventral surface | 2.5 μm above ventral surface |

Serum-deprived (4 h) L6 myotubes were treated with 100 nM insulin for up to 10 min at 37°C. A. After this period cells were fixed, permeabilized and stained for actin (Oregon Green-phalloidin) as described in Materials and Methods. Fluorescent images of actin staining were acquired from cells treated for 0, 3 min, and 10 min with 100 nM insulin. The images are representative of 8 experiments. Scale bar: 10 μm. B. Serial optical sections of 0.5 μm thickness along the Z-axis were generated from myotubes. Optical slices taken at 4 μm (dorsal) and 2.5 μm (ventral) above the ventral surface of myotubes stained for F-actin after insulin treatment are shown. The images are representative of 5 experiments. Scale bar: 5 μm.

The above results revealed that the time course and pattern of insulin-dependent actin filament remodelling (Figure 3.2) was consistent with the dynamics of membrane ruffling observed by scanning electron microscopy (Figure 3.1). Indeed, it is often considered that actin structures support deformabilities of the cell surface, i.e., membrane ruffles. To confirm that the cell surface was remodelled in areas where actin reorganized, a fluorescently-tagged lectin, concanavalin-A (FITC-Con-A) was used to label the cell surface followed by cell permeabilization and labelling of F-actin (rhodamine-phalloidin). In the basal state, Con-A was evenly distributed across the myotube surface (Figure 3.3). Upon insulin stimulation, a marked correspondence was observed between the localization of actin structures (Figure 3.3) and the membrane ruffles (Figure 3.3).

Figure 3.3 Dorsal actin-rich structures cause the membrane ruffling in L6 myotubes

Serum-deprived L6 myotubes were left untreated or stimulated with 100 nM insulin for 10 min at 37°C. The plasma membrane surface was then stained using FITC-conjugated Concanavalin-A (Con-A, 25 μg/ml, 30 min, 4°C), followed by permeabilization and staining for F-actin using rhodamine-phalloidin. Scale bar: 7.5 μm. Arrows indicate regions of membrane ruffling. The images are representative of 3 experiments.
Because muscle cells are enriched in both $\alpha$ (sarcomeric) actin and $\beta$ (ubiquitous) actin, it was necessary to determine which actin isotype was contributing to the formation of the actin structures and membrane ruffling. L6 myotubes were stained for $\alpha$- or $\beta$-actin using specific monoclonal antibodies followed by Cy3-conjugated secondary antibody. Figure 3.4 reveals that $\beta$-actin comprised the majority of F-actin forming the stress fibers in the basal state. Alpha-actin staining was diffuse and appeared globular, and did not label stress fibers (Figure 3.4). Upon stimulation with insulin, $\beta$-actin continued to stain filamentous actin structures which were devoid of any $\alpha$-actin staining (Figure 3.4). These results suggest that 1) $\beta$-actin remolds in response to insulin; and 2) the new actin structures likely result from the remodelling of previously existing actin stress fibers, and not by the polymerization of G-actin into F-actin.

**Figure 3.4** $\beta$-actin, but not $\alpha$-actin forms actin stress fibers which reorganize

Serum-depleted L6 myotubes were treated with or without 100 nM insulin for 10 min at 37°C. Cells were fixed and stained for $\alpha$-actin or $\beta$-actin using actin specific monoclonal antibody, followed by Cy3-conjugated secondary antibody. The images are representative of 3 experiments. Scale bar: 7.5 $\mu$m.

*Effect of Disrupting Actin Filament Formation on Actin Remodelling and Insulin Action*

The four drugs that alter actin organization, cytochalasin D (CD), latrunculin B (LB), jasplakinolide, and swinholide-A, were used to determine their effects on the formation of the actin structures. As described in the Background section, CD, LB and jasplakinolide lead to eventual loss of all actin filaments through depolymerization, whereas swinholide-A selectively interferes with actin filament remodelling, leaving the normal cytoskeleton intact. L6 myotubes were pre-treated with the above agents for the times indicated, then F-actin was stained using rhodamine-phalloidin. Because jasplakinolide occupies the same binding site on F-actin as
phalloidin (50). F-actin was detected in jasplakinolide-treated cells using the β-actin specific antibody. As shown in Figure 3.5, CD and LB completely dispersed actin stress fibers and jasplakinolide caused a shortening and thickening of the stress fibers. Unlike CD, LB, and jasplakinolide, pretreatment of L6 myotubes with swinholide-A did not affect the intensity of F-actin stress fibers in the basal state (Figure 3.5). Upon insulin stimulation of cells where F-actin was depolymerized by CD, LB, or jasplakinolide, actin could no longer remodel into the structures (Figure 3.5). Importantly, when swinholide-A-treated cells were stimulated with insulin, an incomplete formation of the actin mesh was observed. The resulting actin structures were shorter and contained significantly less branching. Therefore swinholide-A prevented the full-fledged remodelling of actin in response to insulin.

**Figure 3.5 Effect of actin filament disrupting compounds on basal and insulin-stimulated F-actin morphology**

<table>
<thead>
<tr>
<th>CON</th>
<th>CD</th>
<th>LB</th>
<th>Jasp</th>
<th>Sw.-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td><img src="image1.png" alt="Basal Images" /></td>
<td><img src="image2.png" alt="Basal Images" /></td>
<td><img src="image3.png" alt="Basal Images" /></td>
<td><img src="image4.png" alt="Basal Images" /></td>
</tr>
<tr>
<td>Insulin</td>
<td><img src="image1.png" alt="Insulin Images" /></td>
<td><img src="image2.png" alt="Insulin Images" /></td>
<td><img src="image3.png" alt="Insulin Images" /></td>
<td><img src="image4.png" alt="Insulin Images" /></td>
</tr>
</tbody>
</table>

L6 GLUT4myc myotubes were exposed to 1 μM CD (2 h), 1 μM LB (1 h), 2 μM jasplakinolide (Jasp, 30 min), or 12.5 nM swinholide-A (Sw.-A, 1 h) during the end of serum deprivation, and were then treated with or without 100 nM insulin for 10 minutes at 37°C. Cells were fixed and stained for F-actin as described in Figure 3.2, or β-actin (for Jasp-treated cells) using β-actin specific monoclonal antibody, followed by Cy3-conjugated secondary antibody. (The control for Jasp-treated cells is in Figure 3.4). The images are representative of 3 experiments. Scale bar: 5 μm.

It was previously shown that complete dispersal of actin filaments with CD or LB inhibits insulin-mediated glucose transport in L6 myotubes (415), 3T3-L1 adipocytes (453, 112, 317), and rat adipocytes (314). The correlation between the morphological and metabolic consequences of actin depolymerization (CD, LB, jasplakinolide) and partial cortical actin
remodelling (swinholide-A) was further confirmed by the densitometric quantitation of surface GLUT4myc in insulin-stimulated cells pre-treated with these agents. The presence of the c-myc epitope tag in the first exofacial loop of GLUT4 allowed for the direct detection of GLUT4 insertion in the plasma membrane in unpermeabilized myotubes (451). CD and LB almost fully blocked insulin-mediated GLUT4myc translocation to the cell surface, whereas interfering with actin turnover by jasplakinolide inhibited 71% \((p<0.01)\) of the insulin response (Figure 3.6). Swinholide-A treatment reduced (62%. \(p<0.01\)) GLUT4myc appearance at the cell surface following insulin stimulation, in parallel to causing the partial inhibition of actin remodelling as observed in Figure 3.5.

**Figure 3.6 Effect of actin filament disrupting compounds on basal and insulin-stimulated GLUT4myc translocation**

L6 GLUT4myc myotubes were exposed to 1 \(\mu\)M CD (2 h), 1 \(\mu\)M LB (1 h), 2 \(\mu\)M jasplakinolide (JASP, 30 min), or 12.8 \(\mu\)M swinholide-A (SW.A, 1 h) during the end of serum deprivation, and were then treated with (INS) or without (BAS) 100 nM insulin for 30 min (C) at 37°C. Quantification of cell surface GLUT4myc was determined as described in Materials and Methods. Results are expressed as the fold-change in cell surface GLUT4myc relative to basal cells in the untreated control (mean \(\pm\) SE of 4 independently performed experiments). *\(p<0.01\), **\(p<0.001\) compared to respective control in the absence of drug. (The results presented for CD and LB were prepared with the assistance of Mr. Atsunori Ueyama.)

**Role of Rho Family G-proteins in Insulin-Dependent Actin Reorganization and GLUT4myc Translocation to the Cell Surface**

Three of the drugs used to prevent actin polymerization in the above experiments (CD, LB, jasplakinolide) resulted in complete dispersal of filamentous actin, making it difficult to conclude whether it was the existence of actin filaments or the remodelling of those filaments that was required for insulin-regulated GLUT4 traffic. On the other hand, swinholide-A caused
partial actin remodelling and inhibited 62% of insulin-dependent GLUT4 traffic, suggesting that the reorganization of F-actin is important for the insulin response. In addition to these pharmacological approaches, a molecular strategy of selectively interfering with insulin-stimulated actin remodelling was attempted. It was previously shown that insulin-dependent membrane ruffling in Swiss 3T3 fibroblasts is mediated by the Rho GTP-binding protein family member, Rac (343). To test a requirement for Rac in the actin remodelling events mediated by insulin, a dominant inhibitory Rac1 mutant, Rac1-N17 (486) was transiently transfected into L6 myotubes (Figure 3.7). Co-transfection of Rac1-N17 cDNA with enhanced green fluorescent protein (EGFP) cDNA was performed to facilitate recognition of transfected cells. Expression of Rac1-N17 did not affect the abundance of long actin stress fibers in either the basal or insulin-stimulated states (Figure 3.7A). However, Rac1-N17 expression prevented the formation of the dorsal actin-rich structures observed previously and in adjacent non-transfected cells (Figure 3.7A). In contrast, transfection of dominant inhibitory Rho (N17) did not affect actin structure formation in response to insulin, but caused a slight irregularity in the appearance of the stress fibers (Figure 3.7B).

The consequence of Rac1 inhibition on the translocation of GLUT4myc to the cell surface was examined by direct detection of GLUT4myc insertion by cell surface immunostaining. In the basal state, cells transfected with Rac1-N17 displayed the same density of GLUT4myc staining on the cell surface as adjacent, untransfected cells in the same optical field (Figure 3.7C). However, the insulin-dependent appearance of GLUT4myc was completely blocked in cells transfected with dominant negative Rac1 (Figure 3.7C). Together, the results in Figure 3.7 strongly suggest that Rac1-dependent actin remodelling is linked to the incorporation of GLUT4-containing vesicles into the plasma membrane in L6 myotubes.
Role of the Actin Cytoskeleton in the Propagation of Insulin Signals from the Insulin Receptor to PI3-K

In this section, the effect of insulin-induced actin remodelling on the distribution of insulin signalling molecules was examined by co-immunostaining the various signalling intermediaries together with F-actin. To ensure that any observed co-localization between actin and any of these proteins was not due to non-specific binding of IgGs to actin structures or to the phalloidin dye, L6 myotubes were stained with antibody against irrelevant IgGs or secondary antibodies alone (Figure 3.8A). In additional control experiments, cells were immunostained for PKCB, a cytosolic protein that is not regulated by insulin treatment, under conditions otherwise described in Figure 3.8A. Neither antibodies against PKC-β (Figure 3.8B), nor control antibodies (Figure 3.8A) colocalized with the actin mesh after insulin treatment.

Figure 3.7 Dominant inhibitory Rac-1, but not Rho blocks insulin-stimulated actin filament remodelling and GLUT4myc translocation to the cell surface

A

EGFP + Rac N17

F-actin

Basal

Insulin

B

EGFP + Rho N17

F-actin

EGFP

Rac N17

Rho N17

C

EGFP + Rac N17

Basal

Insulin

Surface GLUT4-myc

100
L6 GLUT4myc myotubes were transiently transfected with 0.3 µg of enhanced green fluorescent protein (EGFP) cDNA together with 1.2 µg of Rac1-N17 (A,C) or Rho-N17 (B) cDNA. Cells were treated with 100 nM insulin (INS) for 10 min at 37°C. After this period, the cells were either fixed, permeabilized and stained for F-actin (rhodamine-phalloidin, A,B), or processed for detection of GLUT4myc at the cell surface with anti-myc antibody followed by Cy3-conjugated goat anti-mouse antibody (C). For each condition, the green panels show EGFP fluorescence in transfected cells. The red panels show the F-actin staining pattern (A,B) or the cell surface GLUT4myc density (C). Arrowheads indicate the positions of cells transfected with EGFP and Rac1-N17 or Rho-N17. The images are representative of at least 3 experiments. Scale bar, 7.5 µm.

Figure 3.8 PKCβ, and control IgGs do not colocalize with insulin-induced actin structures

![Figure 3.8](image)

L6 myotubes were incubated in the absence or presence of 100 nM insulin for 10 min, followed by fixation and permeabilization. F-actin was labelled with Oregon Green-conjugated phalloidin. A. Insulin-treated myotubes were labelled with non-immune rabbit serum followed by Cy3-conjugated goat anti-rabbit antibody (a); non-immune mouse IgG followed by FITC-conjugated goat anti-mouse antibody (b); Cy3-conjugated goat anti-rabbit antibody alone (c); and FITC-conjugated goat anti-mouse antibody alone (d). B. PKCβ was stained with specific polyclonal antibody, followed by Cy3-conjugated secondary antibody. Scale bar: 5 µm. (The results shown in panel A were prepared with the assistance of Dr. Peter Tong.)

The first polypeptide in the insulin signal transduction cascade examined was the β-subunit of the membrane-bound insulin receptor (IR). Figure 3.9 shows that staining for the β-subunit of the IR did not coincide with the membrane ruffles generated by actin reorganization.

Figure 3.9 The insulin receptor does not collect in insulin-induced actin structures

![Figure 3.9](image)

L6 myotubes were incubated in the absence or presence of 100 nM insulin for 10 minutes, followed by fixation and permeabilization. F-actin was labelled with Oregon Green-conjugated phalloidin. IR was stained with specific polyclonal antibody, followed by Cy3-conjugated secondary antibody. White arrows indicate regions of actin remodeling. Scale bar: 15 µm. (The results shown in this figure were prepared by Dr. Peter Tong.)
Because the docking protein IRS-1 links IR activation to PI3-K, the distribution of intracellular actin filaments and IRS-1 protein was examined by double labelling with Oregon Green-phalloidin and IRS-1 antibody coupled to Cy3-conjugated secondary antibody, respectively. In the basal state, immunostained IRS-1 was diffuse and punctate throughout the myoplasm (Figure 3.10). Insulin treatment resulted in a relocalization of a portion of intracellular IRS-1 to the newly formed actin structures (white arrows in Figure 3.10).

Figure 3.10 A portion of IRS-1 colocalizes with insulin-induced actin structures

L6 myotubes were stimulated with or without 100 nM insulin for 10 min at 37°C, followed by fixation and permeabilization. F-actin was labelled with Oregon Green-conjugated phalloidin. IRS-1 was stained with specific polyclonal antibody, followed by Cy3-conjugated secondary antibody. For each condition, top and bottom panels show the same field of cells. White arrows indicate regions of remodelled actin filaments. The images are representative of 3 experiments. Scale bar: 5 μm.

As the 85 kDa regulatory subunit of PI3-K (p85) couples to tyrosine-phosphorylated IRS-1 upon insulin stimulation, the possibility that PI3-K relocates to the dorsal actin structures was explored next. Comparative analysis of the cellular localization of p85 with actin filaments is illustrated in Figure 3.11A. In the basal state, p85 staining was diffusely punctate throughout the myoplasm, but was more concentrated in the region of the myonuclei, where it appeared to overlap with actin filaments. Within 3 min of insulin stimulation however, a fraction of p85 was found to relocate into the newly formed structures of actin filaments (not shown), becoming most prominent at 10 min of insulin treatment (3.11A). Therefore, a fraction of IRS-1 and p85 staining colocalized with remodelled actin filaments. As described in the Background section, it is well established that binding of the 85 kDa regulatory subunit of PI3-K (p85) to phosphorylated IRS-1 leads to activation of the catalytic subunit of PI3-K (p110), which in turn phosphorylates membrane phosphoinositides. Both the p110α and p110β
isoforms have been implicated in insulin action in various cell types including muscle cells. To determine whether these isoforms of p110 collect with the subset of IRS-1 and p85α in the insulin-induced actin structures, the intracellular localization of p110α and p110β was examined next. Interestingly, under basal conditions, labelling of the p110α polypeptide appeared filamentous and strongly overlapped with the long actin stress fibers (Figure 3.11B). Moreover, upon stimulation of myotubes with insulin, p110α staining continued to mirror F-actin and was markedly redistributed into regions where F-actin remodeled (Figure 3.11B). In contrast to p110α, in unstimulated cells, p110β protein appeared diffusely distributed throughout the cytoplasm in regions devoid of actin filaments (Figure 3.11C). Furthermore, in striking contrast to IRS-1, p85α, and p110α, p110β clearly escaped relocalization into actin structures upon insulin stimulation (Figure 3.11C).

**Figure 3.11 p85α and p110α, but not p110β concentrate in insulin-induced actin structures**

![Image](image-url)

L6 myotubes were stimulated with or without 100 nM insulin for 10 min at 37°C, followed by fixation and permeabilization. F-actin was labelled with Oregon Green-conjugated phalloidin. p85α or p110 proteins were stained with specific polyclonal antibodies, followed by Cy3-conjugated secondary antibody. For each condition, top and bottom panels show the same field of cells. White arrows indicate regions of remodelled actin filaments. The images are representative of 3 experiments. Scale bar in A, 10 μm; scale bar in B, 5 μm; scale in C is the same as in B.

Although a portion of p85α-p110α was observed by fluorescence microscopy to gather in the newly formed actin structures upon insulin stimulation, it is not clear whether this subset of PI3-K within the actin structures represents competent enzyme, capable of generating PI-
It is well-established that both IRS-1 and PI3-K subunits redistribute from the cytosol to intracellular membranes upon insulin stimulation (196, 195). These membranes may conceivably be bound to cortical actin and may provide substrate (PI-4,5-P₂) for phosphorylation by the p85α-p110α PI3-K complex activated by insulin. A fluorescent indicator composed of green fluorescent protein (GFP) fused to the PH domain of the Arf1 exchange factor, general receptor for phosphoinositides (GRPI) was used to visualize whether PI-3,4,5-P₃ is generated in these regions. It was previously shown in vitro that PH-GRPI has a higher affinity (Kᵦ~25 nM) for PI-3,4,5-P₃ over other phosphoinositides, making it an ideal marker for detecting local regions of PI-3,4,5-P₃ production (331). GFP-PH-GRPI cDNA was transiently transfected into L6 myoblasts and cells were co-stained for F-actin using rhodamine-phalloidin. Myoblasts were used instead of myotubes to maximize the efficiency of transfection, and to reduce the expression time to 16 h. We previously showed that myoblasts are insulin responsive (425) and can reorganize actin filaments (333), although the structures are not as prominent as in the much larger myotubes. GFP-PH-GRPI was largely concentrated in the nucleus due to its elevated expression and the highly basic nature of the PH domain (Figure 3.12). However, the protein also demonstrated a cytosolic staining pattern consistent with a scarcity of PI-3,4,5-P₃ in membranes in unstimulated cells (not observable at the exposure shown in Figure 3.12A). Upon insulin stimulation, GFP-PH-GRPI appeared in regions of the cell where active actin remodelling occurred (Figure 3.12, cell designated by open arrow), suggesting that PI-3,4,5-P₃ was formed in membranes colocalizing with the structures. Strikingly, cells highly overexpressing GFP-PH-GRPI (evident by overexposed nuclear signal, closed arrow in Figure 3.12) did not contain any actin structures and their actin morphology resembled that of unstimulated cells. Therefore, overexpression of GFP-PH-GRPI prevented insulin induction of actin reorganization, presumably by sequestering PI-3,4,5-P₃. This result is
consistent with previous findings which showed that the remodelling of actin filaments by insulin requires PI3-K activity (415).

Figure 3.12  PI-3,4,5-P_3 is generated at membrane ruffles, and overexpression of GFP-PH-GRP1 inhibits insulin-stimulated actin remodelling

L6 myoblasts were transiently transfected with 0.5 μg of GFP-PH-GRP1 cDNA for 16 h as described in Materials and Methods. Cells were serum deprived (4 h), then stimulated with or without 100 nM insulin for 10 min at 37°C. After this period, the cells were fixed, permeabilized and stained for F-actin (rhodamine-phalloidin). For each condition, upper and lower panels show the same optical field of cells. The open and closed arrowheads indicate the positions of cells expressing low or high levels of the fusion protein, respectively. The images are representative of 4 experiments. Scale bars, 5 μm.

If PI-3,4,5-P_3 is produced in membranes within the actin structures, then the PI3-K substrate, PI-4,5-P_2 would presumably be localized to the same membrane region. In preliminary studies examining the cellular location of PI-4,5-P_2, we attempted to use GFP fused to the PH domain of PLCδ (GFP-PH-PLCδ) as a probe (380). Transient transfection of GFP-PH-PLCδ into L6 myoblasts for as little as 12 h was deleterious to the cells, likely owing to the importance of PI-4,5-P_2 for many cellular functions. The cells were viable, but were significantly shrunken and rounded up. We detected the GFP-PH-PLCδ probe in reticular membraneous structures throughout the myoplasm in the basal state, and in membrane ruffles in the insulin-stimulated state (results not shown). These preliminary findings require further experimentation, but are at least suggestive that PI3-K can access PI-3,4-P_2 substrate in membranes associated with cortical actin structures.

As reviewed in the Background section, PI3-K activity is essential for triggering GLUT4 externalization in response to insulin. Given that overexpression of GFP-PH-GRP1 prevented insulin-dependent actin remodelling, the role of PI-3,4,5-P_3 in GLUT4 translocation was
examined next in L6 myoblasts stably expressing GLUT4myc. The basal levels of GLUT4myc on the cell surface were slightly lower in cells expressing high levels of GFP-PH-GRP1 (Figure 3.13). Strikingly, the insulin-dependent increase in GLUT4myc density on the cell surface was completely blocked in cells overexpressing GFP-PH-GRP1 (Figure 3.13 middle panels), compared to the density of GLUT4myc staining in adjacent, untransfected cells or cells expressing low levels of GFP-PH-GRP1 (Figure 3.13 right panels). Densitometric scanning of 5 fields of view from at least three experiments was done in order to quantitate the inhibition of GLUT4 translocation caused by overexpressing GFP-PH-GRP1. A value of 100% was assigned to the insulin response above basal in untransfected cells treated with insulin in each field of view [as in (333)]. The pixel intensity of the transfected cells in the same field of view was calculated as a fraction of this value for low or high expressing cells. From this analysis it was determined that insulin-treated cells expressing high levels of the GFP-GRP1-PH construct retained 20.5±3.8% of insulin-stimulated GLUT4myc translocation to the cell surface compared to non-transfected cells. On the other hand, low expressing cells retained 73.7±3.8% of the maximum insulin response. This result is consistent with previous reports showing that pharmacological inhibition of PI3-K using wortmannin prevents insulin-stimulated GLUT4 arrival at the cell surface, and suggests that PI-3,4,5-P3 alone contributes to GLUT4 translocation.

Figure 3.13 Overexpression of GFP-PH-GRP1 prevents GLUT4myc externalization

L6 myoblasts were transiently transfected with 0.5 μg of GFP-PH-GRP1 cDNA for 16 h. Cells were serum deprived (4 h), then stimulated with 100 nM insulin for 20 min at 37°C. After this period, the cells were left intact and processed for detection of GLUT4myc at the cell surface with anti-myc antibody followed by Cy3-conjugated goat anti-mouse antibody. For each condition, top and bottom panels show the same optical field of cells. The open and
Role of the Actin Cytoskeleton in Propagating the Insulin Signal Transduction Pathway From PI3-K to its Effectors

PI-3,4,5-P₃ produced by PI3-K recruits two signalling molecules to cellular membranes, Akt and atypical PKC isoforms (PKCα and PKCζ), both of which have been implicated in insulin-stimulated GLUT4 recruitment to the cell surface. Akt and aPKC are normally cytosolic but associate with purified membranes from insulin-stimulated cells. Because PI-3,4,5-P₃ production was observed proximal to the actin structures, presumably within membranes containing PI3-K, we next explored whether PI3-K effectors (Akt and aPKC) are also drawn into regions of cortical actin remodelling.

Figure 3.14 Akt and phospho-Akt collect in insulin-induced actin-rich structures

L6 myotubes were stimulated with or without 100 nM insulin for 10 min at 37°C, followed by fixation and permeabilization. F-actin was labelled with Oregon Green-conjugated phalloidin. Akt1 (A) or phospho-Akt (Ser 473, B) proteins were stained with specific polyclonal antibodies, followed by Cy3-conjugated secondary antibody. Cells in the bottom, right panel of B were treated with 100 nM wortmannin (WM) for 15 min prior to insulin stimulation. White arrows indicate regions of remodelled actin filaments. The images are representative of 3 experiments. Scale bar: 10 μm.

L6 myotubes were left untreated or stimulated with insulin, and subsequently F-actin and Akt were labelled respectively with Oregon Green-phallolidin and Akt-1 antibody or phospho-specific Akt antibody (Ser 473). The use of the phospho-antibody allowed for the distinction between activated (phosphorylated) Akt and the remaining pool of Akt which was very
abundant in the cytosol. Furthermore, phospho-Akt antibodies recognize all three Akt isoforms (Keene, Sweeney and Klip, unpublished observations). Under unstimulated conditions, Akt-1 staining was diffuse (Figure 3.14A) and the phospho-Akt signal was barely detectable (Figure 3.14B), reflecting the low tonic level of Akt activity in these cells. Insulin stimulation led to an increased intensity of intracellular phospho-Akt labelling which was prevented in cells pretreated with 100 nM wortmannin (Figure 3.14B), confirming that the phospho-antibody reflected an activated cellular state of the enzyme. Notably, a portion of activated Akt molecules prominently colocalized with insulin-induced actin structures (Figures 3.14B). Like phospho-Akt, immunostained Akt-1 also collected in the actin structures (Figure 3.14A).

The observation that a subset of activated PI3-K and Akt colocalized with insulin-induced actin structures is suggestive of a role for F-actin in spatially coordinating the connection between PI3-K and its effector Akt. It is well-established that an intact actin network is not required for insulin signal transduction up to and including PI3-K activation (415, 320). However, when cells were pretreated with CD or LB to depolymerize actin, the ability of insulin to activate Akt was diminished by over 40%, with no observable affect on basal Akt activity (Yaworsky and Klip, unpublished observations). This result suggests that the actin network is required for communication between PI3-K and Akt, possibly via the formation of the actin mesh.

The spatial relationship between actin filaments and the other PI3-K effector, aPKC, was explored next. Because the aPKC isoform involved in insulin-dependent GLUT4 translocation is not definitive, the distribution of both PKCα and PKCζ was explored using isoform-specific polyclonal antibodies. In the basal state, both proteins were diffusely distributed in small puncta throughout the myoplasm and did not colocalize with actin (Figure 3.15A and B). Although aPKC is normally cytosolic in unstimulated conditions, the punctate aPKC staining did not
resemble the more diffuse cytosolic staining of Akt, which is also soluble. Upon insulin treatment, the pattern of both PKCα (Figure 3.15A) and PKCζ (Figure 3.15B) staining did not concentrate to any extent with the newly formed actin-rich structures. Therefore although a portion of one PI3-K effector, Akt, redistributed to the actin structures, the other effector, aPKC, was excluded from this localization.

**Figure 3.15 PKCζ and PKCα (aPKC) do not appear in insulin-induced actin-rich structures**

![Figure 3.15](image)

Serum-depleted myotubes were stimulated with 100 nM insulin for 10 min at 37°C, followed by fixation and permeabilization. F-actin was labelled with Oregon Green-conjugated phalloidin. aPKC proteins were stained with specific polyclonal antibodies, followed by Cy3-conjugated secondary antibody. For each condition, top and bottom panels show the same field of cells. White arrows indicate regions of remodelled actin filaments. The images are representative of 3 experiments. Scale bar in A, 7.5 μm; B, 5 μm.

**Figure 3.16 Disruption of the actin cytoskeleton with CD or LB blocks aPKC activation by insulin**

![Figure 3.16](image)

Serum-depleted L6 myotubes were pre-treated with CD (1 μM, 2 h) or LB (1 μM, 1 h) prior to stimulation with 100 nM insulin for 10 min. aPKC activity was assayed in cell lysates as described in Materials and Methods. The observed kinase activity was normalized relative to the basal activity in untreated cells which was assigned a value of 1. Results are expressed as the mean ± SE of 3 independent experiments. *P<0.005 relative to basal control. (The results presented in this figure were prepared with the assistance of Dr. Gary Sweeney.)

Although aPKC did not appear in the actin structures where PI3-K and Akt localized, the effect of actin depolymerization on aPKC activation by insulin was nevertheless examined. Interestingly, pre-treatment with LB, but not CD, caused a marked increase in basal aPKC.
activity in L6 myotubes (Figure 3.16). Furthermore, upon stimulation of CD- or LB-treated cells with insulin, there was no further increase in aPKC activity relative to CD- or LB-treated basal cells (Figure 3.16). Therefore, dispersal of the actin network inhibited insulin-stimulated aPKC activity, although CD and LB exhibited different effects on basal activity. The reason for the discrepant effects of the two drugs is currently not known.

**Relationship Between GLUT4 Organelles and Remodelled Actin Filaments**

The possible link between actin remodelling and the intracellular localization of the GLUT4 protein was examined. In the basal state, GLUT4myc was concentrated around the myonuclei (Figure 3.17, Basal). Although actin filaments formed substantial dorsal structures within 3 min of insulin treatment, only a small signal of GLUT4myc immunofluorescence began to colocalize with actin structures (Figure 3.17), but became much more pronounced by 10 min of insulin exposure (Figure 3.17). Hence, in contrast to the observations with PI3-K (Figure 3.11), there was a significant delay in the recruitment of GLUT4 protein into the actin structures.

**Figure 3.17 GLUT4 organelles relocate to the dorsal actin-rich structures with a delay following insulin stimulation**

L6 myotubes were stimulated with 100 nM insulin for various times at 37°C, followed by fixation and permeabilization. At 0 min (Basal), 3 min, and 10 min of insulin treatment, actin was labelled with rhodamine-conjugated phalloidin and GLUT4myc was labelled with anti-myc antibody followed by FITC-conjugated secondary antibody. The images are representative of 8 experiments. Scale bar: 7.5 μm. (The results presented in this figure were prepared with the assistance of Dr. Peter Tong.)

The finding that GLUT4myc colocalized with the insulin-induced actin structures (Figure 3.17) indicates that the GLUT4 compartment may be recruited into these regions during
insulin exposure. To confirm this, the staining pattern of the insulin-responsive aminopeptidase (IRAP) was examined. In all insulin-responsive tissues and cell lines studied to date, IRAP colocalizes with GLUT4 in internal membranes, making it a suitable marker for the GLUT4-enriched organelle (388). Under resting conditions, IRAP staining was diffuse and largely perinuclear (Figure 3.18), resembling the GLUT4myc staining pattern (Figure 3.17). At 10 min of insulin stimulation, a significant portion of IRAP redistributed and strongly colocalized with the remodelled actin filaments (Figure 3.18), again reminiscent of the GLUT4myc distribution.

The detection of GLUT4 organelles in the actin structures (Figures 3.17 and 3.18) combined with the earlier observation that the cortical actin structures cause the membrane ruffles (Figure 3.3), raises the possibility that the insertion of GLUT4 into the cell surface occurs at or near portions of the membrane that are distorted by cortical cytoskeletal reorganization. As a first approach to test this hypothesis, insulin-treated L6 GLUT4myc myotubes were double stained for the plasma membrane (FITC-ConA) and for cell surface GLUT4myc. The pattern of distortion of the cell surface observed by Con-A staining demonstrated a close spatial correlation with the appearance of GLUT4myc protein following insulin stimulation (Figure 3.19).
Figure 3.10 The pattern of insulin-induced membrane ruffling parallels the immunostaining of GLUT4myc on the surface of non-permeabilized myotubes

L6 GLUT4myc myotubes were stimulated with 100 nM insulin for 10 min at 37°C, then stained with anti-myc antibody followed by Cy3-conjugated goat anti-mouse antibody to detect cell surface GLUT4myc. Shown are confocal images of FITC-ConA cell surface staining (lower panels) of the cells pictured in the upper panels which were immunostained for cell surface GLUT4myc. Arrows indicate regions of membrane ruffling. The images are representative of 3 experiments. Scale bar: 10 μm.

The finding in Figure 3.19 above suggests that insulin-induced actin remodelling is spatially linked to the incorporation of GLUT4 vesicles into the plasma membrane. However, the resolution of confocal fluorescence microscopy does not allow one to differentiate whether the higher GLUT4myc signal arises from a larger amount of membrane sampled in the ruffled areas. To distinguish between these possibilities, the ultra-structural relationship between surface GLUT4myc protein and membrane ruffles was examined. To this end, the powerful approach of combined backscatter scanning electron microscopy was employed (96). Myc epitopes on the surface of L6 myotubes were labelled immunologically with anti-myc antibody followed by secondary antibody coupled to colloidal 30-nm diameter gold particles and specimens were prepared for scanning electron microscopy. Upon examination of these specimen on the scanning electron microscope, electrons are scattered back by the gold particles. The backscatter imaging mode of the microscope provides an intensity map of the backscattered electron yield from the specimen. Simultaneously, the microcontours of the surface are imaged by using the surface scanning mode (secondary electron imaging). Upon mixing the backscattered electron signal with the secondary electron signal, a high resolution topographical image of cell surface GLUT4myc labelled with colloidal gold particles was obtained. Backscattered electron images revealed all the gold particles as white dots and
permitted the quantitation of the number of gold-labelled epitopes on the cell surface. In unstimulated cells, the cell surface was smooth and there were only a few GLUT4myc molecules on the surface (Figure 3.20A, white dots). Insulin treatment caused a gradual distortion of the dorsal membrane. As in Figure 3.1, there were marked protrusions of the dorsal membrane after insulin treatment (shown in Figure 3.20 at much higher magnification than in 3.1). In association with the membrane ruffles, there was a significant increase in GLUT4myc gold signal (white dots in B and C). The quantitation shown in Figure 3.20D reveals a preferential distribution of gold dots in ruffled areas of insulin-stimulated cells compared to either unruffled areas of the same cells or to any of the (flat) areas in unstimulated cells.

Figure 3.20 Immunogold-labelled cell surface GLUT4 appears in membrane ruffles

<table>
<thead>
<tr>
<th>Basal</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
</tr>
</tbody>
</table>

![Image of immunogold-labeled cell surface GLUT4 appears in membrane ruffles](image)

Figure 3.20D reveals a preferential distribution of gold dots in ruffled areas of insulin-stimulated cells compared to either unruffled areas of the same cells or to any of the (flat) areas in unstimulated cells.
Serum-depleted L6 GLUT4myc myotubes were treated with or without 100 nM insulin for 37°C. Afterwards, the cells were labelled with anti-myc antibody followed by 30 nm gold-conjugated goat anti-mouse antibody to label cell surface GLUT4myc. Specimens were prepared for scanning electron microscopy as described in Materials and Methods. Composite signals of the scanning electron mode and the backscattered electrons from the gold particles (seen as white dots) on the dorsal surface of L6 myotubes are shown after 0 (A), 10 min (B), and 15 min (C) of treatment with 100 nM insulin. White arrows indicate regions of membrane ruffling. White circles encompass samples of white dots. The images are representative of 4 experiments. Scale bar: 1 µm. D: Quantitation of gold particles in ruffled or non-ruffled regions of the cell surface. The number of gold particles was quantitated by applying a 3 x 3 cm grid to the computerized images of control and insulin-stimulated cells from 4 independent experiments, and counting the number of white dots in 5 randomly chosen squares within each grid. *P<0.001 relative to control. (The results presented in this figure were prepared with the assistance of Dr. Peter Tong.)

It was shown earlier that the actin disrupting agents CD, LB and jasplakinolide depolymerized actin and prevented its remodelling (Figure 3.5), and inhibited insulin-stimulated GLUT4 translocation, as detected by densitometric analysis of cell surface GLUT4myc (Figure 3.6). To extend these studies at the ultrastructural level, the effect of disrupting actin filaments with these agents on the insertion of GLUT4myc molecules into plasma membrane ruffles was examined by scanning electron microscopy coupled to backscatter detection of immunogold-labelled myc in non-permeabilized cells. Pre-incubation with CD, LB or jasplakinolide caused a slight distortion of the myotube cell surface and fully prevented insulin-induced formation of membrane ruffles (Figure 3.21). The increased density of cell-surface gold-labelled GLUT4myc caused by insulin was also markedly reduced by these drugs (Figure 3.21, white dots).

Figure 3.21 Ruffle formation and appearance of increased density of immunogold-labelled cell surface GLUT4 is blocked by disrupting the actin cytoskeleton
L6 GLUT4myc myotubes were exposed to 1 μM CD (2 h), 1 μM LB (1 h), or 2 μM jasplakinolide (30 min), and were then treated with or without 100 nM insulin for 10 min at 37°C. Cell surface GLUT4myc was labelled with gold particles and composite signals of the scanning electron mode and the backscattered electrons from the gold particles on the cell surface were obtained as described in Figure 3.18. Scale bar: 1 μm.

**Association of SNARE Proteins with Membrane Ruffles**

Given the close association between GLUT4myc appearance on the cell surface and regions of membrane ruffling, the spatial relationship between actin filaments and the target SNARE proteins syntaxin-4 and SNAP-23 was examined next. Both of these SNARE proteins reside in the plasma membrane and have previously been implicated in the fusion of GLUT4 vesicles with the cell surface (reviewed in Background section). L6 myotubes were left untreated or stimulated for 10 min with insulin, and subsequently the distribution of endogenous syntaxin-4 or SNAP-23 as well as F-actin was examined by confocal microscopy. In the basal state, a portion of SNAP-23 appeared to be filamentous, and already partially colocalized with actin filaments below the cell surface (Figure 3.22A). In contrast, syntaxin-4 was diffusely distributed at the cell membrane and did not colocalize with actin stress fibers in the basal state (Figure 3.22B). Upon insulin treatment, both syntaxin-4 and SNAP-23 staining assumed a pattern in an optical section focused at the dorsal myotube surface (Figure 3.22 A and B). This distribution partially corresponded to the newly formed dorsal actin-rich structures supporting membrane ruffles (correspondence indicated by the white arrows in Figure 3.22A and B).

We next examined whether vesicles containing the v-SNAREs VAMP2 and VAMP3 are drawn into the cortical actin structures induced by insulin since GLUT4 is known to colocalize with a subset of these v-SNARE proteins (reviewed in Background section). L6 myotubes were left untreated or stimulated with insulin, and subsequently endogenous VAMP2 or VAMP3 as well as F-actin were labelled with specific antibodies or phalloidin, respectively. Under unstimulated conditions, both v-SNAREs were localized to a perinuclear region, which was devoid of actin filament staining (Figure 3.23A and B). Upon insulin treatment, a striking
difference in VAMP localization was observed: whereas a distinct VAMP2 signal was detected in the subcortical actin structures (white arrows, Figure 3.23A), VAMP3 clearly escaped such relocation (Figure 3.23B). In L6 myoblasts, which produce smaller cortical actin structures in response to insulin, VAMP2 also colocalized with actin, whereas VAMP3 was absent from the actin structures (shown in (333)), consistent with the phenomenon observed here in myotubes (Figure 3.23).

**Figure 3.22** The t-SNAREs syntaxin-4 and SNAP-23 become concentrated in the insulin-induced membrane ruffles

Serum-depleted L6 GLUT4myc myotubes were stimulated with or without 100 nM insulin for 10 min at 37°C, followed by fixation and permeabilization. Actin was labelled with Oregon Green-conjugated phalloidin. SNAP-23 (A) and syntaxin-4 (B) were stained with specific polyclonal antibodies, followed by Cy3-conjugated secondary antibody. For each condition, top and bottom panels show the same field of cells. Arrows indicate regions of remodelled actin filaments. The images are representative of 3 experiments. Scale bar: 10 µm.

**Figure 3.23** The v-SNARE VAMP2, but not VAMP3 colocalizes with remodelled actin

---

116
Serum-depleted L6 GLUT4myc myotubes were stimulated with or without 100 nM insulin for 10 min at 37°C, followed by fixation and permeabilization. Actin was labelled with Oregon Green-conjugated phallolidin. VAMP2 (A) or VAMP3 (B) proteins were stained with specific polyclonal antibodies, followed by Cy3-conjugated secondary antibody. For each condition, top and bottom panels show the same field of cells. Arrows indicate regions of remodelled actin filaments. The images are representative of 3 experiments. Scale bar: 10 µm.

Detergent Extraction of Insulin Signalling Molecules and Glucose Transporters

The following section examines the segregation of filamentous actin structures with insulin signalling molecules and GLUT4 organelles by biochemical means, using subcellular fractionation and Triton X-100 extraction.

Biochemical Association of Insulin Signalling Molecules with Detergent-Insoluble Complexes

It was recently reported that filamentous structures copurify with internal membranes containing GLUT4, IRS-1, and p85 in 3T3-L1 adipocytes (72). Whether those structures contain actin has not been determined. It was therefore first examined whether actin appears in the corresponding intracellular membrane fractions from L6 myotubes by immunoblotting. Figure 3.24 illustrates the content of actin in the low density microsome (LDM) and cytosolic fractions. Also shown is the distribution of the p85α polypeptide (PI3-K) in these fractions. Table 3.1 demonstrates the relative amounts of these proteins in the two fractions, with the yields of the fractions taken into account. Although the majority of actin in L6 myotubes was cytosolic, a notable actin signal (approximately 20%) was recovered in the LDM fraction (Fig. 3.24 and Table 3.1). Upon insulin stimulation (10 minutes) there was a slight increase in the amount of LDM-associated actin (Figure 3.24), but this was not significant (Table 3.1). By this calculation of cell equivalents, the p85α subunit of PI3-K was also found to be mainly cytosolic, but a detectable amount (approximately 20%) was recovered in the LDM. Insulin decreased the amount of p85α in the cytosol and increased it in the LDM by 30% (p < 0.005, Figure 3.24 and Table 3.1). Interestingly, no p85 polypeptide and very little actin were detectable by immunoblotting of the plasma membrane fraction isolated from either basal or insulin-
stimulated L6 myotubes (not shown). All further biochemical analysis therefore focused on the LDM fraction.

**Figure 3.24 The content of actin and p85 increases in the LDM upon insulin stimulation**

L6 myotubes were treated with 100 nM insulin (INS) for 10 min and subcellular fractionation was performed to purify the low density microsomal fraction (LDM) and cytosolic fraction (CYT). Cell equivalents corresponding to 20% of total cytosol and 50% of total LDM were separated by 10% SDS-PAGE or 7.5% SDS-PAGE and immunoblotting was performed to detect β-actin and p85α, respectively. A gel representative of 5 experiments is shown. Results from these experiments were scanned, quantified and normalized in Table 3.1.

**Table 3.1 Relative amounts of actin and p85 in the cytosol and LDM fraction under basal conditions and in response to insulin stimulation**

<table>
<thead>
<tr>
<th></th>
<th>Actin</th>
<th>p85α subunit of PI3-K</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LDM</td>
<td>Cytosol</td>
</tr>
<tr>
<td>Control</td>
<td>1.0</td>
<td>13.9 ± 0.9</td>
</tr>
<tr>
<td>Insulin*</td>
<td>1.6 ± 0.2</td>
<td>13.1 ± 0.8</td>
</tr>
</tbody>
</table>

Immunoblots of 5 experiments such as the one illustrated in Figure 3.24 were densitometrically scanned, quantitated and corrected for the total cellular yields of each fraction. The total amount of actin or p85 in each fraction is expressed (± SE) relative to the total amount in the untreated LDM, which was arbitrarily assigned a value of 1.0. *Insulin: 100 nM, 10 min. †p < 0.005 relative to control in the absence of insulin.

The observation that p85α, a soluble protein, copurified with actin in the LDM in an insulin-dependent manner suggests that PI3-K may either bind to internal membranes directly or to other proteins associated with the membranes, such as actin. The latter possibility is supported by the observation in Figure 3.11 that p85α and p110α were detected in actin-rich structures induced by insulin. Furthermore, a subset of IRS-1 (Figure 3.10) and Akt (Figure 3.14) proteins also appeared in the actin structures upon insulin stimulation, but not aPKC (Figure 3.15) or p110β (Figure 3.11). The possible association of all of these insulin signalling molecules with the cytoskeleton was examined by analyzing their detergent solubility. The LDM isolated from control and insulin-stimulated L6 myotubes was treated with Triton X-100 and the detergent-soluble supernatant was separated from the insoluble pellet by ultracentrifugation. Both fractions were then immunoblotted to detect actin or the signalling
proteins (IRS-1, p85α, p110α, p110β, Akt, aPKC). Figure 3.25 demonstrates that, with the exception of Akt, all of these proteins in the LDM fraction were insoluble in Triton X-100 in the absence (not shown) or presence of insulin stimulation. Moreover, the majority of immunodetectable β-actin was also detergent-insoluble. Therefore, the Triton X-100-insoluble fraction of the LDM contains a significant fraction of the actin cytoskeleton, although it likely contains many other large insoluble complexes. These findings indicate that all these proteins except Akt are in some way associated with cytoskeletal elements that include actin, however they do not demonstrate that the signalling proteins and actin directly interact with each other.

Figure 3.25 Triton extraction of insulin signalling molecules

Serum-deprived L6 myotubes were treated with 100 nM insulin for 10 min at 37°C and subcellular fractionation was performed to purify the low density microsomal fraction (LDM). A portion (50%) of the total membrane fraction was solubilized with 1% (vol/vol) Triton X-100 and 100 μM DTT for 1 h at 4°C and centrifuged to separate the Triton X-100-soluble supernatant (S) from the detergent-insoluble pellet (P). The resultant fractions were solubilized in sample buffer, separated by SDS-PAGE, and immunoblotted for β-actin (as a control), IRS-1, p85α, p110α, p110β, Akt (pan-antibody recognizing all 3 isoforms) and aPKC (antibody recognizing both λ and ζ isoforms). The gel shown is representative of 4 experiments.

Biochemical Association of Glucose Transporters with the Actin Cytoskeleton

To assess biochemically the possible association of individual GLUT4 and IRAP proteins with the cytoskeleton, LDM and PM derived from control or insulin-treated L6 myotubes were solubilized with 1% Triton X-100 and the Triton-soluble (S) and Triton-insoluble (P) material was recovered (Figure 3.26). In contrast to insulin signalling molecules, which increased in the LDM after insulin treatment (Figure 3.26), GLUT4 and IRAP were decreased in the LDM and increased in the PM. This was expected, due to the rapid redistribution of these proteins to the plasma membrane in response to insulin (388). Strikingly, GLUT4 in the LDM fraction appeared to partition equally between the detergent-soluble
supernatant (S) and detergent-insoluble pellet (P), whereas, all immunodetectable IRAP was recovered in the Triton-soluble fraction, which contained little actin. Furthermore, PM-associated GLUT4 and IRAP remained soluble in Triton X-100 (Figure 3.26). These observations suggest that internal membranes containing the GLUT4 polypeptide, but not IRAP, can associate with the detergent-insoluble material containing the actin cytoskeleton.

To determine whether the observed preference of intracellular GLUT4 to associate with the cytoskeleton was specific to only this GLUT isotype, the detergent solubility of the other two transporters expressed in L6 myotubes (GLUT1, GLUT3) was compared to that of GLUT4 (Figure 3.26). Strikingly, the GLUT1 protein behaved identically to GLUT4, i.e. a portion of LDM-associated GLUT1 was insoluble in Triton X-100, whereas GLUT1 in the PM fraction was fully soluble in the detergent. On the other hand, GLUT3 protein in either the LDM or PM fractions could not be sedimented with Triton-insoluble material.

**Figure 3.26 Triton extraction of GLUT-containing membranes**

Serum-depleted L6 myotubes were treated with or without 100 nM insulin for 10 minutes and subcellular fractionation was performed to purify the low density microsomal fraction (LDM) and the plasma membrane (PM). A portion (50%) of the total LDM or PM derived from basal (BAS) or insulin-treated (INS) cells was solubilized with 1% (vol/vol) Triton X-100 and 100 μM DTT for 1 hour at 4°C and centrifuged to separate the Triton X-100-soluble supernatant (S) from the detergent-insoluble pellet (P). The resultant fractions were solubilized in sample buffer, separated by SDS-PAGE, and immunoblotted for β-actin, IRAP, GLUT4, GLUT1, and GLUT3. The data are representative of 4 experiments.

**DISCUSSION**

**Insulin-Induced Actin Remodelling and GLUT4 Translocation**

This chapter presented a detailed analysis of the morphological changes in actin filaments brought about by insulin and how they might be linked to the metabolic responses of the hormone. Insulin provoked a rapid and marked aggregation of filamentous actin into
structures that projected from the dorsal surface of myotubes causing membrane ruffling. Complete depolymerization of actin filaments with CD, LB or jasplakinolide disrupted all actin architecture and blocked GLUT4 translocation in response to insulin. Interfering with only cortical actin remodelling (but not the longitudinal actin filaments), by pharmacological (swinholide-A) or molecular (dominant negative Rac, GFP-PH-GRP1) means markedly reduced insulin-stimulated mobilization of GLUT4 to the cell surface. Taken together these results suggest that the remodelling of actin filaments is important for recruitment of GLUT4 to the plasma membrane, and support the hypothesis that the actin structures provide the coordinates for the insulin-derived signals to meet the GLUT4 organelles, and direct their insertion into the membrane ruffles.

**Actin as a Scaffold for Insulin Signalling Molecules**

Given the multiple diverse biological functions requiring Class I PI3-K, it is conceivable that a particular subcellular segregation would be involved in each response. The results presented here in L6 myotubes, and previous findings in 3T3-L1 adipocytes revealed that IRS-1, PI3-K, Akt and aPKC rapidly associate with membranes containing the GLUT4-enriched compartment in insulin-stimulated cells. It was suggested that the interaction of IRS-1 and PI3-K with internal membranes involves cytoskeletal elements because, in 3T3-L1 adipocytes, pretreatment of cells with CD precludes the subsequent detection of PI3-K on GLUT4-containing membranes (453), and because insulin resistance releases IRS-1 from cytoskeletal complexes associated with membranes (73). Consistent with these observations, the results presented in this chapter show that LDM-associated IRS-1, p85α, p110α and p110β sediment with detergent-insoluble complexes which are also enriched in cytoskeletal elements (Figure 3.25). Therefore, it is likely that “translocation” of these normally cytosolic proteins to
membranes results from their association with the cytoskeleton, rather than their association with the membranes themselves.

Because p110α appeared tethered to actin filaments even in the absence of insulin (Figure 3.11), p110α may supply the link for the activated IRS-1-p85α-p110α complex to gather within remodelled actin. The inhibition of cortical actin remodeling in cells highly overexpressing GFP-GRP1-PH (Figure 3.12) is consistent with the previous reported inhibition by wortmannin, supporting the notion that PI3-K activation is required for this response (415, 451, 420, 200). Whether the PI-3,4,5-P₃ generated by p110α within the region containing the actin structures (Figure 3.12) is the phosphoinositide required for actin remodelling in the first place can not be determined from our studies. However, the generation of PI-3,4,5-P₃, in membranes gathered by actin structures (Figure 3.12) suggests that the concentration of the IRS-p85α-p110α complex in these regions facilitates the propagation of the PI3-K-dependent signal(s) necessary for the translocation of GLUT4 (see model in Overall Discussion, Figure 6.1). The lack of colocalization of the p110β isoform with the actin structures may indicate that the catalytic activity of p110β is not required for GLUT4 mobilization.

**Role of the Actin Cytoskeleton in Coupling PI3-K to its Downstream Effectors**

The finding that phosphorylated Akt was drawn into the actin structures in insulin-stimulated cells indicates that Akt may be activated in response to PI3-K activity on lipids gathered by the actin mesh. It is not likely that Akt itself lies upstream of actin reorganization because transfection of a dominant negative Akt mutant into L6 myoblasts did not prevent insulin-stimulated actin remodelling (452). Biochemically, Akt protein bound to internal membranes did not sediment with detergent-insoluble material (Figure 3.25), suggesting that Akt itself is not tethered to the cytoskeleton, but rather transiently colocalizes with actin by virtue of its recruitment into actin-associated membrane compartments. The present findings
support the notion that insulin-induced actin structures act as a scaffold for PI3-K and GLUT4 organelles so that PI-3,4,5-P₃, generated within the GLUT4-containing compartment can engage Akt. How Akt activation might lead to GLUT4 insertion into the plasma membrane remains uncertain, although it has been proposed that Akt may phosphorylate proteins on GLUT4 organelles required for vesicle fusion (236).

The hypothesis that the actin cytoskeleton is necessary for the coupling of PI3-K to Akt is supported by two important lines of evidence in L6 myotubes. The first is that actin depolymerization with CD inhibits insulin-stimulated Akt-activity [Tsakiridis et al. unpublished observations; and (320)] and GLUT4 translocation [Figure 3.6 and (415)], but does not affect PI3-K activation (415, 320). Secondly, Akt appeared in PI-3,4,5-P₃-containing membranes within actin structures after insulin stimulation (Figure 3.14), but was not itself tethered to cytoskeletal complexes (Figure 3.25). Taken together, these results suggest that the major breakdown in insulin signalling caused by actin disruption occurs at a step between PI3-K activation and Akt activation. Indeed, several models of insulin resistance show reduced Akt activity and GLUT4 translocation, in the absence of any effect on the magnitude of insulin-stimulated PI3-K activity, but rather due to mislocalization of PI3-K, making the enzyme unavailable to activate Akt in the proper cellular location (410, 152, 295). This possibility is discussed in detail at the end of the Discussion section for this chapter [point (b) below].

Interestingly, although aPKC is activated downstream of PI3-K and is putatively involved in GLUT4 delivery to the plasma membrane, neither isoform of the enzyme was detected in the actin structures (Figure 3.16). On the other hand, unlike Akt, aPKC proteins were biochemically insoluble in Triton X-100, suggesting that these proteins associate with other cytoskeletal elements. It was previously shown that aPKC binds to the cytoskeleton in T cells in culture (138). The punctate staining pattern of cytosolic aPKC indicates that the protein
may not be truly 'soluble', and is perhaps associated to macrostructures that are resistant to detergent extraction. These detergent-insoluble complexes may be caveolae or may contain other cytoskeletal elements such as microtubules or intermediate filaments. Because depolymerization of actin with CD or LB completely prevented aPKC activation by insulin, it is possible that the insoluble complexes require an actin filament network to properly localize aPKC for activation. It is likely, then, that an actin-independent PI3-K signalling complex may trigger the activation of aPKC required for a different aspect of GLUT4 translocation from Akt such as insertion into the plasma membrane. Indeed there is a recent report of a role for aPKC in phosphorylating VAMP2, a SNARE protein important for GLUT4 vesicle incorporation into the plasma membrane (44).

**Relation of Actin Cytoskeleton Remodelling to Localization of GLUT4 Organelles and their Insertion into the Plasma Membrane**

Actin-based traffic of organelles and vesicles has been reported in many organisms. It is therefore conceivable that reorganization of the subplasmalemmal actin network reflects events that are necessary to allow exocytic GLUT4 vesicles to fuse with the plasma membrane during stimulation by insulin. This chapter explored the extent and nature of the colocalization of proteins of the GLUT4 compartment with the actin cytoskeleton. A portion of intracellular (perinuclear) GLUT4myc and IRAP became concentrated in the newly formed actin-rich structures at the dorsal surface of the myotube (Figures 3.17 and 3.18). These observations suggest that GLUT4 vesicles, which contain both proteins, are recruited to cortical actin structures in response to insulin, and that this actin mesh may directly participate in intracellular traffic of GLUT4-containing vesicles. The presence of an exofacial c-myc epitope tag on GLUT4 allowed for the identification of the site of incorporation of the protein into the plasma membrane. Combining scanning electron microscopy with immunogold labelling of the c-myc epitope provided ultrastructural evidence that translocation and fusion of GLUT4 may
preferentially occur in ruffled regions of the membrane. Indeed, a quantitatively higher GLUT4myc immunogold signal was detected in the ruffles compared to non-ruffled regions of the cell surface of insulin-stimulated cells (Figure 3.20). Immunofluorescence of non-permeabilized cells confirmed that surface GLUT4 proteins spatially corresponded to areas of ruffles (Figure 3.19). The observation that the FITC-ConA signal was also stronger in the ruffles suggest that there is indeed more membrane area in those regions. It is tempting to speculate that insulin-dependent formation of cortical actin structures may target the ongoing fusion of a subgroup of intracellular GLUT4 vesicles with the plasma membrane, and that this contributes to the expansion of the membrane allowing the formation of membrane ruffles. These findings are consistent with other recent observations that GLUT4-containing vesicles are targeted to and fuse at specific loci on the plasma membrane of fat cells (317, 307).

**Does the GLUT4 Polypeptide Interact with the Actin Cytoskeleton?**

It was previously shown that immunopurified GLUT4-containing compartments are also enriched with the actin-binding protein, spectrin (415), suggesting a possible interaction between the actin-based membrane cytoskeleton and the vesicles that supply the cell surface with glucose transporters. Preliminary reports of immunocytochemical colocalization of muscle spectrin isoforms with GLUT4 compartments in L6 myotubes have also been made (86, 85). Taken together, these studies suggest that the actin cytoskeleton may bind to GLUT4-containing vesicles via spectrin, but do not provide any evidence of a direct tethering of GLUT4 to the cytoskeleton. In the basal state, GLUT4 did not colocalize with the long actin stress fibers to a significant extent (Figure 3.17). However, biochemically, a portion of the intracellular GLUT4 was detergent-insoluble in the absence or presence of insulin (Figure 3.26). Only upon insulin stimulation did a portion of GLUT4 colocalize with the emerging cortical actin-rich structures, while the residual staining remained perinuclear (Figure 3.17). Interestingly, all PM-associated
GLUT4 was detergent-soluble (3.26). Furthermore, a fraction of the GLUT1 protein in the LDM was Triton-insoluble, whereas GLUT3 present in the LDM or PM fraction was not cytoskeleton-associated. The question emerges: is a fraction of the intracellular (but not PM) GLUT4 and GLUT1 tethered to the actin cytoskeleton before and after exposure to insulin? Several recent reports support the notion that glucose transporters interact with cytoskeletal elements: a) The GLUT4 C-terminal domain interacts with aldolase, which in turn links to F-actin in unstimulated 3T3-L1 adipocytes (186); b) The GLUT4 carboxyl terminus binds to a myosin-like peptide in cell extracts from unstimulated adipose cells (249); c) GLUT4 tagged with GFP was static and thought to be tethered to an intracellular structure in the resting state in 3T3-L1 adipocytes (307); d) Immuno-isolated GLUT4-containing endomembranes bind to microtubules (313), as does an isoform of the Leishmania glucose transporter (370); e) Two novel proteins were recently reported to link GLUT4 membranes to the actin cytoskeleton: an EH-domain-containing protein termed EH-2 (144) and an insulin-responsive unconventional myosin I isoform, MiMlb/Myr2 (43). Unlike GLUT4, IRAP derived from LDM of L6 myotubes was not found in the detergent-insoluble pellet (Figure 3.26), even though the protein was shown to concentrate with the actin-rich structures by immunofluorescence (Figure 3.18). This last finding suggests that actin remodelling induced by insulin recruits GLUT4-containing compartments, of which IRAP is a resident protein to actin filaments. GLUT4 has the capacity to tether either directly or indirectly to the actin filaments but IRAP does not. This model may additionally explain the ability of CD, LB or jasplakinolide to prevent the redistribution of GLUT4 from internal compartments to the surface of L6 myotubes (Figure 3.6).

**Relationship of SNARE Proteins to Actin Structures**

Three non-neuronal SNARE proteins have been implicated in the fusion of GLUT4 containing vesicles with the plasma membrane following insulin treatment in muscle cells and
adipocytes. These are VAMP2, syntaxin-4 and SNAP-23. Unlike VAMP2, VAMP3/cellubrevin is not required for insulin-dependent GLUT4 vesicle fusion (333), although both proteins have been reported to coexist on GLUT4-containing vesicles. Consistent with this observation, a portion of VAMP2 colocalized with the insulin-induced cortical actin structures, whereas immunostaining for VAMP3 continued to be diffuse and perinuclear. We interpret these results to suggest that VAMP2 and VAMP3 populate distinct vesicles and that only insulin-responsive GLUT4 membranes containing VAMP2 gather into the actin mesh in response to insulin. Their cognate t-SNARE proteins, SNAP-23 and syntaxin-4 are present in the plasma membrane where SNAP-23 mediates the formation of a complex between syntaxin-4 and VAMP2 that is required for the incorporation of GLUT4 into the plasma membrane. It is conceivable that remodelling of actin filaments may facilitate the interaction between plasma membrane t-SNAREs and v-SNAREs on the GLUT4 vesicles. We therefore explored whether insulin-induced actin remodelling could modulate the spatial distribution of the t-SNAREs syntaxin-4 and SNAP-23. Under basal conditions, syntaxin-4 did not show any colocalization with F-actin (Figure 3.22), whereas SNAP-23 appeared as filamentous structures within the cell and at the cell surface, and it partially colocalized with F-actin stress fibers. This is consistent with previous observations that a fraction of the cellular complement of SNAP-23 sediments with detergent-insoluble material containing cytoskeletal elements (120). Following insulin stimulation, a portion of both SNAP-23 and syntaxin-4 became prominent in the membrane ruffles. The presence of t-SNARE proteins and GLUT4 vesicles in membrane ruffles may allow for efficient interactions between t-SNAREs and v-SNAREs on the GLUT4 compartment leading to fusion of GLUT4-containing vesicles with the plasma membrane.
Impairment of Insulin Action by Perturbation of Actin Filaments

Prolonged pre-exposure of L6 myotubes to high glucose and high insulin, a strategy previously shown to render adipose cells insulin resistant, impairs insulin-stimulated glucose uptake. Insulin resistant cells also demonstrated incomplete actin remodelling after acute stimulation with insulin (413). This effect is strikingly similar to the partial inhibition of actin filament reorganization (Figure 3.5) and GLUT4myc translocation (Figure 3.6) caused by swinholide-A. Furthermore preincubation of L6 myotubes with high glucose and insulin markedly prevents Akt activation (Huang and Klip, unpublished observations). These observations raise the question of which events in the insulin-triggered glucose transport response might be involve the actin cytoskeleton. Recent evidence points to four potential cytoskeleton-dependent steps:

(a) Coupling of the insulin receptor with its downstream substrate, IRS. It was recently observed that prolonged exposure of 3T3-L1 adipocytes to high insulin releases insulin receptor substrate (IRS) from a cytoskeletal complex, leading to an inability of the insulin signal to propagate beyond the insulin receptor (73).

(b) The communication of PI3-K with its target signalling molecules. The actin cytoskeleton appears to concentrate PI3-K (p85α-p1 10β) in specific cellular compartments in L6 myotubes (Figure 3.11) and 3T3-L1 adipocytes (453). Moreover actin filament disruption prevents the generation of 3-phosphoinositides and Akt/protein kinase B (PKB) activation (320). Thus, the inhibition of cortical actin remodelling may limit the access of PI3-K products to their specific downstream targets, important for insulin-stimulated GLUT4 translocation. This possibility is supported by recent reports of impaired signalling downstream of PI3-K in various cellular and animal models of insulin resistance (410,152,295,309).

(c) The arrival and fusion of GLUT4 vesicles at the plasma membrane. As described in the Background section, there have been several recent reports of microtubule and intermediate
filament cytoskeletal networks serving as tracks for GLUT4-containing endomembranes (112, 143, 317, 117, 313, 72). Given the intimate interplay between actin and the other cytoskeletal networks, it is possible that disturbance of any of the cytoskeleton during insulin resistance may cause a coordinate disruption of all three networks, leading to inappropriate targeting of GLUT4-containing compartments. It is conceivable that the incompletely formed cytoskeletal architecture may no longer be capable of providing the scaffolding for v-SNAREs on the GLUT4-containing vesicles to appose t-SNAREs in ruffled regions of the cell surface in response to insulin.

CONCLUSIONS

In conclusion, insulin-mediated actin reorganization modulates the redistribution of insulin signalling molecules (IRS-1, p85, p110, and Akt). GLUT4-containing vesicles endowed with IRAP, and VAMP2 to cortical actin structures below the dorsal cell surface. The data suggest that the newly formed actin-rich structures provide the scaffold whereby signalling intermediaries. GLUT4-containing vesicles and SNARE proteins interact to facilitate the insertion of GLUT4 to selective loci (ruffles) in the plasma membrane. The integrity of the actin cytoskeleton is essential for the full insulin-induced translocation of GLUT4 to the cell surface, and incomplete remodelling of F-actin causes insulin resistance of GLUT4 translocation. The actin-dependent compartmentalization of signalling molecules. GLUT4 vesicles and the SNARE fusion machinery may contribute to the metabolic actions of insulin in target tissues such as skeletal muscle.
CHAPTER 4: MEDIATORS OF ACUTE GLUCOSE TRANSPORT REGULATION IN RESPONSE TO METABOLIC DEMAND

The majority of this chapter is based on the following publications:


SUMMARY

The purpose of the studies performed in this chapter was to use a mitochondrial uncoupler, dinitrophenol (DNP), as a tool to investigate possible mediators of the metabolic demand signalling pathway leading to increased glucose uptake. DNP uncouples the mitochondrial oxidative chain from ATP production, thereby preventing oxidative metabolism. The consequent increase in energy demand is, however, contested by the cell by increasing glucose uptake to produce ATP via glycolysis, reminiscent of the response to hypoxia in vivo. In L6 skeletal muscle cells, DNP rapidly causes a doubling in glucose transport. Unlike the insulin response, glucose transport stimulation by DNP is wortmannin-insensitive, and does not require an intact actin cytoskeleton. This chapter reveals that DNP-stimulated glucose uptake was additive to insulin's. DNP was not able to activate enzymes in the insulin signalling cascade (PI3-K, Akt/PKB, or p70S6 kinase). However chelation of intra- and extracellular Ca²⁺ with BAPTA-AM (1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester) in conjunction with EGTA (ethyleneglycol-bis-(α-aminoethyl ether)-N,N,N',N'-tetraacetic acid) inhibited DNP-stimulated glucose uptake and GLUT4 translocation to the cell surface by 80%.

Since conventional protein kinase C (cPKC) can be activated by Ca²⁺, the possibility that cPKC may be activated in response to DNP, and may participate in DNP action in L6 myotubes was also examined. Acute DNP treatment led to translocation of cPKCs to the plasma membrane. Furthermore, cPKC immunoprecipitated from plasma membranes exhibited a 2-fold increase in kinase activity in response to DNP. Overnight treatment with 4-phorbol-12-myristate-13-acetate (PMA) downregulated cPKC isoforms α,β, and γ (but not αPKC) and reduced DNP, but not insulin-stimulated glucose uptake. Consistent with this result, the PKC inhibitor bisindolylmaleimide (BIM-I) blocked PKC enzyme activity at the plasma membrane.
(100%) and inhibited DNP-stimulated [3H]2-deoxyglucose uptake and GLUT4 translocation by 60% with no effect on the stimulation of glucose transport by insulin. Finally, the selective PKCβ inhibitor LY379196 partially inhibited the effects of DNP on glucose uptake (45-60% inhibition).

Recent evidence points to a role for the fuel-sensing enzyme AMPK in hypoxia- and contraction-stimulated glucose transport in skeletal muscle. The final section of Chapter 4 began to explore the participation of AMPK in DNP-stimulated glucose uptake. The chemical AICAR (5'-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside), is taken up and metabolized by cells to form the monophosphorylated AICAR (ZMP), an AMP analog which activates AMPK. The stimulation of glucose uptake or GLUT4 translocation by AICAR was very modest (<25% above basal), and was not additive to the effects of insulin or DNP. However, DNP robustly activated AMPK (3-fold) when in vitro AMPK activity was measured. Therefore, it is not clear whether AMPK participates in DNP-stimulated glucose uptake in L6 myotubes. Taken together, the results suggest that interfering with mitochondrial ATP production acts upon a signal transduction pathway independent from that of insulin, involving Ca2+ and cPKC, but the participation of AMPK is not certain.

RESULTS

DNP-Stimulated Glucose Transport is Additive to Insulin

For all experiments described herein, cells were stimulated with a dose of 0.5 mM DNP, previously shown to maximally stimulate glucose uptake in L6 myotubes (23). The time course in Figure 4.1 demonstrates that the maximal effects of DNP and insulin on glucose transport were partly additive over a 60 min time period after stimulation, reminiscent of previous observations of additivity between insulin and hypoxia, and insulin and exercise, in skeletal
muscle (296, 480). These observations suggest that different signals may participate in relaying the signal from insulin and from DNP to the glucose transporters.

**Figure 4.1 DNP-stimulated glucose uptake is additive to insulin**

L6 myotubes were serum-deprived for 4.5 h, then incubated for 0 to 60 min in α-MEM with either 0.5 mM DNP (○), 100 nM insulin (□) or both (●) at 37°C. Immediately following these incubations the cells were washed twice with HBS and glucose uptake was assayed as described in Materials and Methods. Transport rates were normalized with respect to the basal rate of the untreated control, which was assigned the value of 1. Shown is a representative of 3 independently-performed experiments. Results are expressed as the mean ± SE of three replicates.

**DNP does not Engage the Insulin Signal Transduction Pathway**

Three kinases rapidly activated by insulin in muscle cells are PI3-K (417), Akt/PKB (protekinase B) (218), and the serine kinase p70 S6 kinase (p70 S6K), which is involved in mitogenic responses to the hormone (116). Previous work from this laboratory showed that the selective PI3-K inhibitor, wortmannin, does not affect the activation of glucose transport by DNP in L6 muscle cells, but abolishes the insulin response (418). However, PI3-K activity was not directly measured in that study. Here it is shown that unlike the hormonal response of L6 muscle cells, DNP did not activate PI3-K (Figure 4.2, panel A). It is widely held that insulin-mediated Akt/PKB and p70 S6K activation occurs downstream of PI3-K and is dependent on the lipid products of PI3-K (5, 64), yet recent reports have uncovered stress-induced activation of Akt/PKB [in COS-7 cells (223)] and arsenite-induced activation of p70 S6K [in cardiomyocytes, (455)], which are both PI3-K-independent. We therefore tested whether DNP
activates either Akt/PKB or p70 S6K directly. As shown in Figure 4.2, DNP did not activate either Akt/PKB (panel B) or p70 S6K (panel C) in L6 myotubes.

**Figure 4.2 DNP does not activate lipid and protein kinases activated by insulin**

Serum-depleted myotubes (4.5 h) were treated with 0.5 mM DNP (•) or 100 nM insulin (○) for up to 30 min. PI3-K (A), Akt/PKB (B) or p70 S6K (C) kinase activity was assayed in cell lysates as described in Materials and Methods. The observed kinase activities were normalized relative to the basal activities in untreated cells which were assigned a value of 1. Results are expressed as the mean ± SE of 3 independent experiments.

**Role of Intracellular Ca\(^{2+}\) in DNP-Stimulated Glucose Uptake**

There is evidence that mitochondrial uncoupling with agents such as DNP rapidly increases intracellular Ca\(^{2+}\), which is thought to arise from depletion of ER or mitochondrial Ca\(^{2+}\) stores. This rise in Ca\(^{2+}\) coincides with an acceleration of muscle glucose flux in muscle cells (75). To ascertain the demand for Ca\(^{2+}\) in the activation of glucose transport by DNP, L6 muscle cells were loaded with the calcium chelator BAPTA-AM incubated simultaneously with EGTA. BAPTA is rendered cell-permeant by its acetoxymethylester (AM) which can enter cells, but once inside esterases remove the methyl esters to create a Ca\(^{2+}\) chelator that will not exit the cells. Also, EGTA pretreatment is required to trap extracellular Ca\(^{2+}\). Chelators were added to L6 myotubes prior to challenge with DNP or insulin [as in (210)], and then \[^{3}H\]2-deoxyglucose uptake was measured. Chelation of intra- and extracellular Ca\(^{2+}\) with BAPTA/EGTA inhibited DNP-stimulated glucose uptake by 79% (p<0.01), without affecting
insulin-stimulated glucose uptake (Figure 4.3). Buffering extracellular Ca\(^{2+}\) with EGTA alone did not affect the DNP response.

**Figure 4.3 Role of Ca\(^{2+}\) in the response to DNP**

Serum-depleted myotubes (4.5 h) were pretreated with or without 15 \(\mu\)M BAPTA-AM and 2.5 mM EGTA (BAPTA/EGTA) or 2.5 mM EGTA alone in HBS supplemented with 10 mM D-glucose for 10 min, followed by stimulation for 30 min with 0.5 mM DNP (filled bars) or 100 nM insulin (INS, cross-hatched bars) in HBS. Cells were washed twice with HBS and glucose uptake was measured as described in Materials and Methods. Transport rates were normalized with respect to the basal rate of the untreated control (CON) which was assigned a value of 1. Results are expressed as the mean ± SE of 5 independent experiments. (*p<0.01 vs. respective control)

**Role of Ca\(^{2+}\)-Sensitive PKC in DNP Action**

A rise in intracellular Ca\(^{2+}\) triggers the activation of a variety of cellular proteins, including Ca\(^{2+}\)-sensitive PKC (cPKC) [reviewed in (303)]. To assess the involvement of cPKC in the glucose transport response, the potent PKC inhibitor bisindolylmaleimide (BIM-I) was utilized. BIM-I inhibits Ca\(^{2+}\)-dependent conventional PKC isoforms at lower doses (<1 \(\mu\)M), but higher doses (5-10 \(\mu\)M) also inhibit novel or atypical isotypes, which do not require Ca\(^{2+}\) for activation (263). At 1 \(\mu\)M. BIM-I caused a 61% (p<0.05) reduction in the stimulation of glucose transport by DNP (Figure 4.4) but it did not significantly affect the response to insulin until a higher dose of BIM-I was used (10 \(\mu\)M). At this BIM-I concentration, no additional inhibition of DNP-stimulated glucose uptake was observed, however, insulin-stimulated glucose transport was inhibited by 50%, consistent with evidence for the involvement of aPKC in the insulin-dependent glucose transport pathway in L6 myotubes (18).

Phorbol esters such as 4\(\beta\)-Phorbol 12\(\beta\)-Myristate 13\(\alpha\)-Acetate (PMA) are routinely used to activate PKC in cells and study its function. When added exogenously, PMA binds to the
DAG-binding region of cPKC and anchors the enzyme to the membrane thereby increasing PKC activity. In insulin-responsive cells, PMA is able to stimulate glucose uptake (212) and glucose transporter translocation (204), albeit to a lesser extent than insulin or other stimuli. To confirm the action of 1 μM BIM-I is on cPKC, we tested the effect of BIM-I (1μM) on PMA-stimulated glucose transport in L6 myotubes. Figure 4.5 demonstrates PMA increased glucose transport by 30% and 1 μM BIM-I inhibited the stimulation by 100%, whereas DNP-dependent glucose uptake was partially inhibited (60%) by the same treatment. BIM-I had no effect on the basal value of glucose transport (Figure 4.4 and 4.5).

Figure 4.4 DNP-stimulated glucose transport is reduced by the PKC inhibitor BIM-I

Serum-depleted L6 myotubes (4.5 h) were pre-incubated for 15 min in αMEM in the absence or presence of the indicated doses of BIM-I. The cells were then stimulated for 30 min at 37°C with 0.5 mM DNP (O) or 100 nM insulin (□) in the continuous presence of BIM-I. At the end of this period, the cells were washed twice with HBS and glucose uptake was measured. Transport rates were normalized with respect to the basal rate of the untreated control (UNT), which was assigned the value of one. Results are expressed as the mean ± SE of 4 experiments.

Figure 4.5 BIM-I specifically inhibits phorbol ester-induced glucose uptake

Serum-depleted L6 myotubes (5 h) were pre-incubated for 15 min in α-MEM in the absence or presence of 1 μM BIM-I. The cells were then stimulated for 30 min at 37°C with 0.5 mM DNP, 100 nM insulin (INS), or 1 μM PMA in the continuous presence of BIM-I. At the end of this period, the cells were washed twice with HBS and glucose uptake was measured. Transport rates were normalized with respect to the basal rate of the untreated control (BAS), which was assigned the value of 1. Results are expressed as the mean ± SE of 6 independent experiments. (*p<0.05 vs. respective control in the absence of BIM-I)

The prolonged activation of cPKC elicited by long-term exposure of cells to phorbol ester ultimately leads to their degradation. Thus, downregulation with phorbol ester is a
convenient method of selectively removing cPKC isoforms from a system. To further clarify the dependence of cPKC in DNP-dependent glucose transport activation. cPKCs were depleted from L6 cells by overnight PMA treatment. This treatment eliminated all cPKC isoforms but not the atypical PKC-ζ (Figure 4.6. panel A). Furthermore, cPKC downregulation partially inhibited the stimulation of glucose transport by DNP by 45%, whereas it fully blocked PMA-stimulated glucose uptake (Figure 4.6. panel B). PKC downregulation did not affect the stimulation of glucose transport by insulin (panel B). The findings for insulin and PMA were consistent with previous observations reported by Bandyopadhyay et al. in L6 muscle cells (18).

![Figure 4.6 cPKC downregulation reduces DNP-stimulated glucose transport](image)

L6 myotubes were incubated for 16 h in the absence or presence of 100 nM PMA (downregulated). A. Cells were lysed and various PKC isoforms were detected in lysates by SDS-PAGE followed by immunoblotting. B. Cells were stimulated for 30 min with 0.5 mM DNP, 100 nM insulin or 1 μM PMA. At the end of this period, the cells were washed and glucose transport was assayed. Transport rates were normalized with respect to the basal rate of the untreated control (UNT) which was assigned a value of 1. Results are expressed as the mean±SE of 5 independent experiments. (*p<0.05 vs. respective control)

Although the above findings implicate cPKC in DNP-stimulated glucose uptake, they do not prove that the enzyme is even activated in the presence of DNP. The extent of cPKC activation by DNP was therefore ascertained by two approaches: PKC translocation and kinase activity measurement. The first approach involved assaying cPKC translocation to the plasma
membrane, which is an index of activation of the enzyme (see Background section). Fractions enriched in plasma membranes (PM) derived from DNP-treated L6 cells were purified, followed by immunoblotting for conventional isoforms of PKC with an antibody that recognized α,β, and γ isoforms. DNP generated a 2.6-fold increase in cPKC levels in the PM compared to unstimulated cells (Figure 4.7, panel A, and quantitated in B). PMA on the other hand, provoked a marked redistribution of cPKC to the PM (9-fold), while insulin treatment resulted in only a modest increase in the PM levels of cPKCs. As expected, cPKC could not be detected in PM fractions isolated from cells pretreated with PMA overnight regardless of whether they were stimulated with insulin, DNP or PMA for 30 min (Figure 4.7).

**Figure 4.7 DNP increases translocation of cPKC to plasma membrane**

![Diagram showing translocation of cPKC](image)

Serum-depleted L6 myotubes (4.5 h) were stimulated with 0.5 mM DNP, 100 nM insulin (INS) or 1 μM PMA in α-MEM at 37°C for 30 min. Subcellular fractionation was performed to obtain a plasma membrane-enriched fraction (PM). Equal amounts of PM (50 μg) were separated by 7.5% SDS-PAGE, and immunoblotted for cPKC. A representative immunoblot is shown in A and the quantitation is in B. The results in B are expressed relative to the basal levels of PKC in the plasma membrane from the untreated cells (UNT). ( * p<0.05, **p<0.001 vs. untreated control)

The second approach involved measuring *in vitro* cPKC activity directly in PM fractions derived from unstimulated, DNP-, insulin-, or PMA-stimulated cells. PKC-α,β, and γ activity in cPKC immunoprecipitates from plasma membranes was elevated by 200% by DNP (Figure 4.8, panel A). This activation was completely blocked by pretreatment of cells with 1 μM BIM-
I. Comparable with its stimulation of cPKC translocation to the PM, PMA also induced a much greater activation of cPKC activity (7-fold) compared to DNP (Figure 4.8, panel B), whereas insulin elevated cPKC activity by only 30% (panel A).

**Figure 4.8 DNP induces a cPKC activity that is inhibitable by BIM-1**

Serum-depleted myotubes (4.5 h) were treated with (filled bars) or without (open bars) 1 µM BIM in α-MEM for 15 min prior to stimulation with 0.5 mM DNP or 100 nM insulin (INS, A) or 1 µM PMA (B) for 10 min at 37°C. The cells were fractionated to obtain a plasma-membrane enriched fraction (PM). Conventional PKC was immunoprecipitated from the PM using a monoclonal antibody against cPKC. The kinase activity of the immunoprecipitated PKC on an exogenous substrate was measured. The observed PKC activity was normalized relative to the basal activity in the untreated cells (UNT). The results are expressed as the mean ± SE of 4 to 6 independent experiments. (*p<0.05, **p<0.001 vs. untreated control).

The availability of a selective PKC-β inhibitor, LY379196 (IC<sub>50</sub> ~ 5 nM) (175), allowed for the participation of this isoform in DNP-stimulated glucose transport to be tested. As shown in Figure 4.9, pretreatment of L6 cells with the LY379196 compound partially inhibited DNP-stimulated glucose uptake (45-60%) at an effective concentration of the inhibitor for PKC-β inhibition.

**GLUT4 Translocation also Depends on Ca<sup>2+</sup> Mobilization and cPKC**

To confirm that the observed effects of Ca<sup>2+</sup> chelation and cPKC inhibition of DNP-stimulated glucose uptake resulted from impaired glucose transporter translocation, we utilized the L6 cells stably transfected with a GLUT4 protein with an exofacial myc-epitope tag (L6
GLUT4myc (182, 451). These cells were treated with or without DNP along with various manipulations of Ca\(^{2+}\) or cPKC, and myc-tagged GLUT4 was detected on the cell surface of intact L6 muscle cells. Comparable to the effects on 2-deoxyglucose uptake, pretreatment with either Ca\(^{2+}\) chelation, BIM (1 \(\mu\)M), or cPKC downregulation decreased the GLUT4myc at the cell surface to 37\%, 47\%, and 46\% of the maximal DNP response, respectively (Figure 4.10). As in wild-type L6 muscle cells, DNP-stimulated glucose uptake was also inhibited in L6 GLUT4-myc cells by pretreatment with BAPTA/EGTA. 1 \(\mu\)M BIM, or cPKC downregulation to 19\%, 43\% and 27\% of the maximal DNP response.

**Figure 4.9** Inhibition of PKC\(\beta\) reduces DNP-stimulated glucose transport

Serum-depleted L6 myotubes were pre-incubated for 15 min in \(\alpha\)-MEM containing the indicated doses of the PKC\(\beta\) specific inhibitor LY379196. The cells were then stimulated for 30 min with 0.5 mM DNP (O) in the continuous presence of the inhibitor. At the end of this period, the cells were washed and glucose uptake was measured. Transport rates were normalized with respect to the basal rate of the untreated control (UNT), which was assigned the value of one. Results are expressed as the mean \pm SE of 3 independent experiments. \(*p<0.01\) vs. untreated control

**Figure 4.10** DNP-stimulated GLUT4myc translocation depends on Ca\(^{2+}\) and cPKC
L6 GLUT4myc cells were grown to the stage of myotubes and were pretreated with BAPTA-AM/EGTA (BAP/EGTA), 1 μM BIM (BIM), or were depleted of cPKC (DR) as described in the figure legends for Figures 4.3, 4.4, and 4.6, respectively, prior to treatment with 0.5 mM DNP for 30 min. The DNP-stimulated increase in GLUT4myc translocation (A) or [3H]2-deoxyglucose uptake (B) was assigned a value of 100%. Inhibition of this value towards basal (0%) was calculated after inhibition with BAP/EGTA, BIM, or DR. Results are expressed as the mean ± SE of 3 independent experiments.

**3-O-Methylglucose Uptake also Depends on Ca**²⁺ **Mobilization and cPKC**

The uptake of 2-deoxyglucose is the sum of transmembrane transport and phosphorylation of glucose inside the cell by hexokinase. It has been previously shown that when measuring the transport rate, changes in 2-deoxyglucose uptake reflect changes in the transport step under the assay conditions used. Nonetheless, the effect of ATP depletion by DNP on hexose uptake may have either triggered the stimulation of hexose transport, or modulation of the activity of hexokinase, the enzyme that phosphorylates 2-deoxyglucose to form 2-deoxyglucose-6-phosphate. To ensure that incubation with DNP brings about a response of glucose transport specifically, the uptake of 3-O-methylglucose (a nonphosphorylatable analog of glucose) was also measured. Table 4.1 shows that DNP increased 3-O-methylglucose uptake by about two-fold relative to control cells. Furthermore, pretreatment with BAPTA/EGTA, BIM-I, or cPKC downregulation partially inhibited DNP-stimulated 3-O-methylglucose uptake (Table 1.1). The extent of reduction in DNP-stimulated glucose uptake closely paralleled the results using 2-deoxyglucose uptake as the transported sugar (Figures 4.3, 4.5, and 4.6).

**Table 4.1. DNP also stimulates 3-O-methylglucose uptake in a Ca²⁺ and cPKC-dependent manner**

<table>
<thead>
<tr>
<th>3-O-Methylglucose Uptake (relative to basal in untreated control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>1.00 ± 0</td>
</tr>
<tr>
<td>BAPTA/EGTA</td>
</tr>
<tr>
<td>1 μM BIM</td>
</tr>
<tr>
<td>10 μM BIM</td>
</tr>
<tr>
<td>Downregulated</td>
</tr>
</tbody>
</table>

L6 myotubes grown in 6-well plates were pretreated with BAPTA-AM and EGTA, or with 1 or 10 μM BIM-I, or were depleted of cPKC (downregulated) as described in Figures 4.3, 4.4, and 4.5, respectively, before treatment with 0.5 mM DNP for 30 min. DNP-stimulated increase in 3-O-[methyl-²H]methylglucose.
uptake was assayed as described in *Materials and Methods*. Transport rates were normalized with respect to basal rate of untreated control, which was assigned a value of 1.00. Values are means ±SE of 3 independently performed experiments.

**Effect of Combining Ca\(^{2+}\) Chelation and cPKC Inhibition on the DNP Response**

Because a residual increase in glucose uptake was observed after chelating intracellular Ca\(^{2+}\) (~20%, Figure 4.3) or inhibiting cPKC (~40%, Figure 4.4), it was next examined whether the cPKC-dependent portion of glucose uptake was part of the Ca\(^{2+}\)-dependent component. To do this, Ca\(^{2+}\) chelation was combined with cPKC inhibition. Table 4.2 shows that the effect of Ca\(^{2+}\) buffering on DNP action was not enhanced by simultaneous cPKC inhibition or cPKC depletion.

**Table 4.2 Combination of cPKC inhibition and Ca\(^{2+}\) chelation does not further inhibit the DNP response**

<table>
<thead>
<tr>
<th>2-Deoxyglucose Uptake (pmol·min(^{-1})·mg(^{-1}))</th>
<th>Basal</th>
<th>DNP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>4.57 ± 0.37</td>
<td>9.91 ± 0.62</td>
</tr>
<tr>
<td><strong>B/E</strong></td>
<td>4.20 ± 0.12</td>
<td>5.19 ± 0.32</td>
</tr>
<tr>
<td><strong>B/E + 1 µM BIM</strong></td>
<td>4.52 ± 0.23</td>
<td>5.41 ± 0.16</td>
</tr>
<tr>
<td><strong>B/E + Downregulated</strong></td>
<td>4.16 ± 0.18</td>
<td>5.26 ± 0.15</td>
</tr>
</tbody>
</table>

After no treatment or incubation with 1 µM BIM-I for 20 min, or 100 nM PMA for 16 h (downregulated), L6 myotubes were pretreated with 15 µM BAPTA-AM and 2.5 mM EGTA (B/E), in Ca\(^{2+}\)-free HBS supplemented with 10 mM D-glucose for 10 min. After this period, cells were stimulated for 30 min with 0.5 mM DNP in HBS. Cells were washed twice with HBS and 2-deoxyglucose uptake was assayed. Values are means ± SE of 3 replicates. Experiment shown is representative of 3 independently performed experiments.

**Role of AMPK in DNP Action**

The possible participation of AMPK in the DNP-stimulated glucose uptake was briefly considered. The compound AICAR is metabolized in skeletal muscle tissue into ZMP which is an AMP analog able to activate AMPK. DNP- or AICAR-treated skeletal muscle exhibits a markedly elevated activation of AMPK (151). In contrast, in L6 myotubes, chemically activating AMPK by treating L6 myotubes with AICAR (2.5 mM), caused only a slight (<25%) increase in glucose uptake (Table 4.3). Even when AICAR levels were brought to 10 mM, no
greater effect on glucose uptake was observed (not shown). Furthermore, the small increase in glucose uptake elicited by AICAR was not additive to insulin or DNP-stimulated glucose uptake (Table 4.3). These results suggest that, unlike in skeletal muscle, AICAR metabolism in cells in culture does not lead to glucose transport elevation.

**Table 4.3 AICAR does not stimulate glucose uptake in L6 GLUT4myc myotubes**

<table>
<thead>
<tr>
<th>2-Deoxyglucose Uptake (pmol/min/mg protein)</th>
<th>Basal</th>
<th>AICAR</th>
<th>DNP</th>
<th>INS</th>
<th>DNP+AICAR</th>
<th>INS+ AICAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>4.38 ± 0.12</td>
<td>5.13 ± 0.16</td>
<td>8.28 ± 0.17</td>
<td>8.48 ± 0.19</td>
<td>8.67 ± 0.16</td>
<td>8.14 ± 0.10</td>
</tr>
</tbody>
</table>

L6 myotubes were treated with 2.5 mM AICAR alone or in the presence of 0.5 mM DNP or 100 nM insulin (INS) for 30 min. The rate of [3H]2-deoxyglucose uptake was measured. Results are representative of 3 independent experiments.

To determine whether AMPK is truly activated during exposure of L6 myotubes to DNP, AMP activity on an exogenous substrate was measured using an *in vitro* kinase assay. The time course in Figure 4.11 shows that DNP activates AMPK to at least 3-fold above basal within 10 min of stimulation. Therefore, the AMPK enzyme is activated during metabolic demand in L6 myotubes.

**Figure 4.11 DNP robustly activates AMPK in a time-dependent manner**

Serum-starved (4.5h) L6 myotubes were stimulated with DNP (0.5 mM) for various time periods. Cells were lysed then assayed for AMPK activity as described in *Materials and Methods*. Results were normalized relative to basal activity in untreated cells, which was assigned a value of 1. Results are expressed as the mean ± SE of three individual experiments. *P < 0.01 (Results for this figure were obtained with the assistance of a summer student, Mr. Nish Patel).
DISCUSSION

Distinct Glucose Transport Stimulation Pathways Exist

Several lines of evidence suggest that different pathways exist for the stimulation of glucose transport into skeletal muscle by insulin compared to energy demand (see Background section). For example, a combination of the two stimuli produces an additive effect on stimulation of glucose uptake (323, 296, 480). Furthermore, insulin signalling requires activation of PI3-K and Akt/PKB but hypoxia and contraction do not (248, 254). The results presented in Figure 4.1 in this chapter show that the stimulation of glucose uptake by DNP in L6 muscle cells is additive to that induced by insulin. Also, unlike insulin, DNP does not activate PI3-K, Akt/PKB or p70 S6K (Figure 4.2). Similarly, muscle contraction has no effect Akt/PKB activity (254). Our findings support the notion that at least two distinct pathways leading to the stimulation of glucose uptake also exist in L6 muscle cells.

The Calcium/PKC Hypothesis

It has long been considered that the rise in intracellular Ca²⁺ is a critical mediator of increased glucose transport during skeletal muscle contraction and hypoxia (60, 164). Indeed, Ca²⁺ is released from mitochondria as a result of DNP dissipation of the H⁺ gradient. It is therefore possible that Ca²⁺ may be a trigger in the insulin-independent mechanism of glucose transport activation. The findings presented in Figure 4.3 with the buffering of intra- and extracellular Ca²⁺ provide more direct evidence that Ca²⁺ plays a significant role in the stimulation of glucose transport induced by DNP, but not by insulin.

A rise in cytoplasmic Ca²⁺ levels may facilitate the activation of key intracellular signalling molecules that lead to increased muscle glucose transport. PKC is a Ca²⁺-dependent signalling intermediary that can be activated by increases in cellular Ca²⁺. Because Ca²⁺ can activate cPKC's, and PMA (a known activator of cPKC) can increase glucose transport by a
mechanism distinct from insulin (18, 134, 212, 442), the participation of cPKC in DNP-stimulated glucose transport was an intriguing possibility. The results in this chapter suggest that DNP, acting through Ca\(^{2+}\)-sensitive PKC, can modify L6 muscle cell glucose transport based on four lines of evidence: a) The downregulation of conventional, but not atypical PKC protein isoforms, decreased DNP-stimulated glucose transport by 45%, with no effect on insulin-induced glucose uptake (Figure 4.6); b) The DNP-induced rise in glucose transport was lowered by 60% by a low dose of BIM (1 μM) which is known to effectively inhibit cPKC, whereas the insulin response was only affected at a far greater BIM-1 concentration; c) DNP caused a rapid translocation to and activation of PKC-α, -β, and -γ at the cell surface; d) Using LY379196 to selectively inhibit PKC-β, we observed a partial decrease (approx. 45-60%) in the stimulation of glucose transport by DNP (Figure 4.9). Notably, BIM-1 and downregulation of cPKC reduced transport rates by 60% and 45%, respectively. Therefore, although PKCβ may participate in glucose transporter mobilization during metabolic challenge, it may not necessarily account for all of the cPKC-mediated activation of glucose transport. Previous reports have revealed PKC activation during muscle contraction (76, 340); however the role of PKC remained controversial since it was never determined which of the 12 different PKC isoforms could be responsible for this effect. If specific antagonists for the other Ca\(^{2+}\)-sensitive PKC isotypes become available, it will be possible to identify the specific cPKC mediators of the alternative mechanism of glucose transport activation.

As PMA-stimulated glucose transport was completely inhibited by 1 μM BIM-1 and by PMA-downregulation of cPKC (overnight), yet no more than 60% of the stimulation by DNP was inhibited by these manipulations, there may be a PKC-independent component to the stimulation of glucose uptake by DNP. Conversely, PMA stimulates cPKC activity by 8-fold but is able to induce only a 50% rise in glucose transport at best. Therefore, robust activation of
PKC alone is not sufficient to increase glucose transport to levels comparable to those induced by DNP or insulin. Discrepant effects of phorbol esters, insulin and hypoxia on glucose transport have been noted previously (134, 147, 442).

Using L6 GLUT4myc cells, it was shown in Figure 4.10 that the inhibition of DNP-stimulated [¹H]2-deoxyglucose uptake caused by Ca²⁺ chelation or by interfering with cPKC activation is reflected by a decrease in the mobilization of GLUT4myc to the cell surface. GLUT4myc translocation, assessed by a colorimetric detection assay, was impaired by these manipulations to nearly the same extent as glucose transport in wild type L6 or L6 GLUT4-myc muscle cells. Therefore, the effect of cPKC inhibition/downregulation and Ca²⁺ buffering on DNP-stimulated glucose transport occurred at a signalling step proximal to GLUT4 translocation rather than at the level of vesicle docking/fusion or by directly affecting glucose transporter activity. Consistent with a role for PKC stimulation of GLUT4 vesicle translocation, numerous early studies report that agents that activate PKC can stimulate exocytosis in a variety of cell types (166, 181). Billiard et al. observed that the exocytosis of secretory vesicles in rat pituitary gonadotrophes could be stimulated independently by either Ca²⁺ elevations or by PKC activation with PMA (36). As the stimulation of glucose uptake by DNP involves incorporation of GLUT-containing vesicles into the plasma membrane, the participation of cPKC in this step is a distinct possibility.

**Is AMPK a Mediator of the Metabolic Demand Pathway?**

Ca²⁺ chelation was more effective than cPKC inhibition in reducing the DNP stimulation of glucose uptake. However, even this treatment left a residual increase in glucose uptake. Also, the effect of Ca²⁺ buffering on DNP action was not enhanced by simultaneous cPKC inhibition or cPKC deletion (Table 4.2). Assuming that all treatments were equally effective on their targets (i.e., they fully inhibited cPKC and prevented rises in cytoplasmic Ca²⁺, as appropriate),
then it is possible that three types of signals cooperate to bring about the DNP effect on glucose uptake: cPKC activation, a secondary effect of Ca\(^{2+}\), and a Ca\(^{2+}\)-independent signal. It is likely, then, that cPKC is only one component in the signalling downstream of Ca\(^{2+}\) in the activation of glucose transport by DNP. The rapid drop in ATP levels that occurs within minutes of DNP exposure may signal the activation of additional molecules participating in the stimulation of the glucose uptake system in muscle cells. Logically, a candidate mediator is AMPK. Indeed, AMPK has received considerable consideration as a potential signalling molecule involved in the alternative pathway because it is activated by various conditions that lower the fuel status of the cell and its activation correlates with glucose uptake in several systems studied (151, 92, 237, 34, 348). We show here that DNP also robustly activates the enzyme (Figure 4.12). although chemical activation of AMPK with AICAR was unable to stimulate glucose uptake (Table 4.3). In follow-up studies performed in collaboration with a student in our lab, we showed that AICAR, when added to L6 cells, does not even activate the enzyme (Patel and Klip, unpublished observations). These further studies also revealed that AMPK could be fully activated in two conditions (BAPTA/EGTA or BIM-I pre-treatment) even though glucose uptake was significantly blunted (316). These results are consistent with a recent report of a lack of correlation of AMPK activation and contraction-stimulated glucose uptake in skeletal muscle (98), and a lack of AICAR-stimulated glucose uptake in CHO cells expressing G\(_i\) receptor, although AMPK is activated (206). Thus, whether AMPK participates in the alternative pathway or is merely activated by metabolic stress in parallel to other mediators remains unclear.

CONCLUSIONS

In summary, the findings presented suggest that DNP may employ Ca\(^{2+}\) as a secondary messenger to activate conventional PKCs, forming part of the metabolic demand signalling
system leading to the regulation of glucose transport in L6 muscle cells. This alternative pathway functions independently of the PI3-K signalling pathway utilized by insulin to increase muscle cell glucose influx, and may not involve AMPK.
The majority of the work presented in this chapter was published in:


---

INTRODUCTION AND SUMMARY OF THE STUDY

The previous chapter explored differences between DNP and insulin in the acute stimulation of glucose transport in L6 myotubes. This chapter deals with the chronic action of DNP on glucose transport. Because information on this mechanism was not included in the Background section, this summary includes the pertinent background information on chronic glucose transport regulation.

The increase in glucose uptake caused by energy stressors and insulin in L6 muscle cells occurs in two stages. The initial phase (associated with a redistribution of pre-formed glucose transporters to the cell surface) reaches a maximum within 15-20 min of stimulation and is normally maintained for up to 60 min (462). In the prolonged presence of these activators, renewed effects can be observed as early as 90-120 minutes with a further increase in glucose uptake beyond the acute phase. This increase continues for up to 24 h reaching a maximum of 4-fold, and is known to result from an elevated cellular protein content of glucose transporters. Long-term (18 h) exposure to insulin or to disruption of oxidative phosphorylation with DNP triggers an increased rate of glucose transport associated with an elevation in the protein content of GLUT1 and GLUT3, but not GLUT4 (23, 398), suggesting that GLUT4 is mostly engaged in the acute response. It was previously shown that the rise in GLUT1 expression likely results from de novo biosynthesis of the transporter since the uncoupler increases GLUT1 mRNA levels. In contrast, very little is known about how interfering with mitochondrial ATP production regulates GLUT3 protein expression.

Given the distinct mechanisms by which DNP and insulin acutely regulate glucose transport, it could be reasoned that differences in glucose transport regulation occur during the chronic phase of stimulation as well. Therefore in this Chapter, the mechanisms employed by DNP to increase GLUT3 protein content and glucose uptake in L6 muscle cells was examined. The effects on GLUT3 protein and mRNA levels of prolonged exposure of L6 myotubes to
DNP in comparison to the effects on GLUT1 expression were explored, and were also compared to the response elicited by insulin. Continuous exposure to DNP had no effect on GLUT3 mRNA levels, but elevated GLUT1 mRNA. Moreover, DNP-stimulated glucose transport was unaffected by the protein-synthesis inhibitor cycloheximide. The increase in GLUT3 protein mediated by DNP was also insensitive to cycloheximide, paralleling the response of glucose uptake, but the rise in GLUT1 protein levels was blocked by the inhibitor. The GLUT3 glucose transporter may therefore provide the majority of the glucose transport stimulated by DNP despite elevated levels of GLUT1 protein. The half-lives of GLUT3 and GLUT1 proteins in L6 myotubes were determined to be about 15 h and 6 h, respectively. DNP markedly prolonged the half-life of GLUT3 protein. These results suggest that the long-term stimulation of glucose transport by DNP arises from an elevation of GLUT3 protein content associated with an increase in the GLUT3 protein half-life. These findings indicate that disruption of the oxidative chain of L6 muscle cells leads to an adaptive response of glucose transport that is distinct from the insulin response, involving specific glucose transporter isoforms that are regulated by different mechanisms.

RESULTS

**Time Course of GLUT3 and GLUT1 Induction by DNP**

For all experiments described herein, cells were stimulated with a dose of 0.5 mM DNP, previously shown to maximally stimulate glucose uptake in L6 myotubes (23). Cell viability was unaffected at this dose for up to 24 h. The exposure of L6 myotubes to 0.5 mM DNP caused a rapid and progressive increase in glucose uptake, that peaked at 8 h and was sustained for up to 24 h (Figure 5.1). From the time course in Figure 5.1, 18 h was chosen to be the optimal time point for long-term treatment with DNP.
Figure 5.1 Time course of DNP-stimulated glucose transport

L6 myotubes were treated with 0.5 mM DNP for the indicated time period, with all incubations initiated so that each experimental group was incubated for the entire 18 h time period before further processing. [3H]-2-deoxyglucose uptake was determined. Glucose transport in untreated cells (time = 0 h) was assigned a value of 1.0, and all other values were expressed in relative units. Each data bar represents the mean ± SE for three independently performed experiments.

Incubation with DNP for 18 h elevates the content of GLUT3 and GLUT1 glucose transporters in L6 myotubes (23, 398), yet the steady-state level of GLUT4 protein is not altered (398). To determine the time course of the induction of GLUT3 and GLUT1, Western blot analysis of the glucose transporter proteins was performed on total membrane fractions from L6 cells incubated for up to 18 h in the presence or absence of 0.5 mM DNP (Figure 5.2 A and B). Representative immunoblots of GLUT3 and GLUT1 are shown in panel A and the results from 4 experiments are summarized in panels C and D. The DNP-mediated increases in GLUT3 and GLUT1 followed similar time courses. There was a slow phase of increase of GLUT3 and GLUT1 followed by steep increases in both isoforms after 6 to 8 h of treatment with DNP. However, the effect of DNP on GLUT1 at this time was more pronounced than its effect on GLUT3. By 18 h of exposure to DNP, GLUT3 and GLUT1 proteins were elevated by 3- and 5-fold, respectively (Figure 5.2).

GLUT3 and GLUT1 mRNA Response to DNP

Chronic insulin causes an elevation in levels of GLUT3 and GLUT1 mRNA in L6 muscle cells, leading to increased expression of the corresponding proteins (23, 260, 398). To test whether the same phenomenon occurs with prolonged DNP treatment, Northern blot analysis was performed using total RNA extracted from L6 muscle cells treated for 18 h with or
without 0.5 mM DNP. Interestingly, and in contrast to insulin (positive control, Figure 5.3), GLUT3 mRNA levels were unaltered by treatment of L6 myotubes with DNP for 18 h (Figure 5.3, panel A). However, continuous exposure to DNP led to an increase in steady-state levels of GLUT1 mRNA, similar to the effects of insulin (Figure 5.3, panel B). Therefore, although DNP increased GLUT3 protein levels, it did not increase the steady-state levels of GLUT3 mRNA.

**Figure 5.2** Time course of DNP-dependent elevation of GLUT3 and GLUT1 steady-state protein levels

L6 myotubes were treated with 0.5 mM DNP for the indicated time period. with all incubations initiated so that each experimental group was incubated for the entire 18 h time period before further processing. Total membrane fractions from each experimental group were immunoblotted for GLUT1 (A) and GLUT3 (B). To ensure equality of protein loading, all membranes were also probed for the α1-subunit of the Na+-K+-ATPase, a membrane protein whose expression is not altered with continuous exposure to DNP (398) (not shown). Representative immunoblots for time-dependent changes of GLUT3 and GLUT1 in response to DNP are shown. C and D: Immunoblots were quantified and the results are expressed as the mean ± SE of 4 independent experiments. Content of GLUT3 protein (●) or GLUT1 (○) from untreated cells was assigned a value of 1.0, and all other values were expressed in relative units. [*,# Indicates statistically significant compared to untreated control (*, p<0.001; #, p<0.05)]

**Figure 5.3** Steady-state GLUT3 mRNA is not elevated by DNP treatment

Total RNA was isolated from L6 myotubes incubated with 0.5 mM DNP or 100 nM insulin (INS) for 18 h, as described in Material and Methods. Twenty micrograms of RNA were analyzed by Northern blotting using a GLUT3 (A) or GLUT1 (B) riboprobe. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was probed for a loading control (C). The Northern blots of 4

<table>
<thead>
<tr>
<th>Steady-State GLUT mRNA Level</th>
<th>Control</th>
<th>DNP</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT3</td>
<td>1.00±0.0</td>
<td>0.86±0.14</td>
<td>3.61±0.47</td>
</tr>
<tr>
<td>GLUT1</td>
<td>1.00±0.0</td>
<td>4.11±0.26</td>
<td>4.42±0.23</td>
</tr>
</tbody>
</table>
independent experiments were quantified, and their values are expressed relative to the untreated control, given the value of 1.00.

**Effect of Cycloheximide on Glucose Transporters and Glucose Transport**

To assess the requirement for ongoing protein synthesis in the response of increased glucose transport and transporter content, L6 muscle cells were treated with the protein synthesis inhibitor cycloheximide for the duration of DNP challenge. With DNP treatment of L6 myotubes, GLUT3 protein levels remained elevated during coincubation with 2 μg/ml cycloheximide, while the GLUT1 protein increase was completely blocked by the inhibitor (Figure 5.4). For comparison, the effects of cycloheximide on insulin-mediated GLUT3 and GLUT1 induction were also examined. In contrast to the uncoupler, the insulin-stimulated elevation in GLUT3 protein was fully prevented by cycloheximide as was GLUT1 protein (Figure 5.4). The low concentration of cycloheximide used in these experiments (2 μg/ml) did not directly inhibit or activate glucose transport since basal levels of transporters (Figure 5.4) and glucose uptake (Figure 5.5, see below) were not affected by the drug.

**Figure 5.4 Induction of GLUT3 protein levels by DNP treatment is insensitive to cycloheximide**

Differentiated L6 cells were preincubated for 60 min in the presence (open bars) or absence (filled bars) of 2 μg/ml cycloheximide (CHX) followed by subsequent incubation with 0.5 mM DNP or 100 nM insulin (INS) for 18 h with or without the continuous presence of CHX, as required. Total membrane fractions were prepared and subjected to immunoblot analysis. To assess equality of protein loading, all Western blot membranes were also probed for the α1-subunit of the Na⁺-K⁺-ATPase whose expression is not altered with continuous exposure to DNP or insulin (35, 398) (not shown). Representative immunoblots are shown for GLUT3 and GLUT1 from DNP- (A) and insulin-treated cells (C) and quantified in B (GLUT3) and D (GLUT1) as the mean ± SE of 4 independently performed experiments. [*p<0.0001, ** p<0.05 vs. basal; # p<0.0001, ## p<0.05 vs. respective control in the absence of cycloheximide*]
As shown in Figure 5.5, the majority of glucose transport in response to chronic DNP treatment remained elevated in the presence of 2 μg/ml cycloheximide, yet the insulin-stimulated glucose transport was completely blocked. Thus in the face of cycloheximide inhibition of protein synthesis, the DNP-stimulated increase of GLUT3 protein was paralleled by continued elevation of glucose transport.

**Figure 5.5 DNP-stimulated glucose transport is insensitive to cycloheximide**

Differentiated L6 cells were preincubated for 60 min in the presence (open bars) or absence (filled bars) of 2 μg/ml cycloheximide (CHX) followed by subsequent incubation with 0.5 mM DNP or 100 nM insulin (INS) for 18 h with or without the continuous presence of CHX, as required. Uptake of 2-deoxyglucose was measured. Each data bar represents the mean ± SE for triplicate determinations within a representative experiment, independently performed three times. Where error bars were not noticeable, errors were too small to be printed. [*p<0.0001 compared with basal; # p <0.0001 vs. respective control in the absence of cycloheximide]*

**Effect of DNP on the Half-Life of Glucose Transporters**

To assess whether post-translational mechanisms are responsible for the sustained elevation of GLUT3 protein mediated by DNP, we examined the effects of the uncoupler on the rate of degradation of glucose transporter proteins. L6 myotubes were metabolically labelled with [15S]methionine for 4 h, a time sufficient to substantially label most cellular proteins (results not shown). After chasing for the indicated times with media containing unlabelled methionine in the presence or absence of 0.5 mM DNP, the amount of label associated with glucose transporter immunoprecipitates was determined. Under basal conditions, the profiles of the decay of radiolabel of the two GLUT isoforms were clearly different. In untreated cells the GLUT3 label decreased by 25% by 8 h of chasing and dropped to 75% by the 24 h chase period (Figure 5.6, panel A). In contrast, the labelled GLUT1 rapidly dropped by 75% within 8 hours of chase time and remained at this level until the 24 h chase period (Figure 5.6, panel B). The half-lives of GLUT3 and GLUT1 in L6 myotubes were calculated to be 15 h and 6 h,
respectively. The half-life of GLUT3 was markedly prolonged by DNP. More than 80% of the radioactive label remained associated with GLUT3 immunoprecipitates after 24 h of exposure to DNP. In contrast, the half-life of GLUT1 rose only slightly with DNP treatment, although a striking effect of DNP treatment was observed at the 8 h time point (Figure 5.6, panel B). The actual change in half-lives could not be directly calculated since it would require more than 24 h of chasing in the presence of DNP, which compromises the viability of L6 muscle cells. Nevertheless, the results in Figure 5.6 clearly show that the degradation of GLUT3 was significantly inhibited by DNP.

**Figure 5.6** Long-term DNP treatment leads to an increase in the half-life of GLUT3 protein

L6 myotubes were pulsed and chased in the absence (\(\square\), CON) or presence of 0.5 mM DNP (\(\bullet\)). Glucose transporters were then immunoprecipitated and separated by 10% SDS-PAGE. The labelled GLUT3 and GLUT1 immunoprecipitates were quantified by Phosphorimager analysis as shown in A (GLUT3) and B (GLUT1). Results are expressed as the percentage of \(\text{\textsuperscript{35}S}\) label remaining in glucose transporters immunoprecipitated at 0 h of chase time and represent means ± SE of 6 independent experiments. Pre-immune complexes and immune supernatants contained no immunologically detectable glucose transporters (results not shown). Statistics: Significantly higher than untreated control (\(*p<0.0001\); \#p<0.01).

As a first approach to understand what cellular mechanism is responsible for the normal degradation of GLUT3 in L6 myotubes, the lysosome was targeted, as it is a major cellular protein degradation pathway. Lysosomal proteases were inhibited using a cell-permeable form of the protease inhibitor, E-64, termed E-64d. This compound is an irreversible inhibitor of cysteine proteases in the lysosome (402). In preliminary experiments, exposure of L6 myotubes to E-64d for 18 h caused an increase in steady-state levels of both GLUT1 and GLUT3 proteins (Figure 5.7). This result suggests that the lysosome is the major pathway for degradation of
these proteins, and interfering with ATP production may downregulate lysosomal degradation of GLUT3.

**Figure 5.7 Effect of pharmacological inhibition of lysosomal proteases on steady-state GLUT1 and GLUT3 protein levels**

L6 myotubes were left alone (CON) or incubated with 0.5 mM DNP, 100 nM insulin (INS) or 4 μM E-64d for 18 h. Total membrane fractions were prepared and subjected to immunoblot analysis. Representative immunoblots from 2 replicate experiments are shown for GLUT3 and GLUT1.

**Do Other Forms of Hypoxia Increase GLUT3 Expression by the Same Mechanism as DNP?**

Given the surprising finding of the isoform-specific post-translational regulation of GLUT3 expression during prolonged DNP treatment, the possibility that this regulation is common to all forms of chronic metabolic stress was next examined. The battery of metabolic insults tested were rotenone (mitochondrial inhibitor), cobalt chloride (CoCl₂, sequesters heme from O₂), and hypoxia (3% O₂ chamber). The effects on glucose uptake and GLUT levels after exposure of myotubes to 100 μM CoCl₂, 0.5 μg/ml rotenone, or to a hypoxic chamber for 18 h were measured. All three insults caused increases in glucose uptake (Figure 5.8), and GLUT1 and GLUT3 protein content (Figure 5.9), to levels comparable to the response elicited by DNP. Surprisingly, in the presence of the protein synthesis inhibitor cycloheximide, only rotenone behaved like DNP. That is, the ability of rotenone to increase GLUT3 protein levels and glucose uptake was not blocked by cycloheximide, whereas GLUT1 induction was reduced (Figures 5.8 and 5.9). On the other hand, the increases in GLUT1, GLUT3 and glucose uptake triggered by hypoxia and CoCl₂ were all inhibited when protein synthesis was prevented (Figures 5.8 and 5.9). These results suggest that directly uncoupling or inhibiting mitochondrial ATP production for several hours delivers different signals towards GLUT regulation than preventing O₂ uptake by the mitochondria.
Figure 5.8 Protein synthesis inhibition with cycloheximide does not affect DNP and rotenone-stimulated glucose uptake but blocks the response to hypoxia and CoCl₂

L6 myotubes were treated with DNP (0.5 mM), insulin (100 nM), CoCl₂ (0.1 mM, "Cobalt"), rotenone (0.5 μg/ml) or incubated in a hypoxic chamber (3% O₂) for 18 h, in the presence (filled bars) or absence (open bars) of the protein synthesis inhibitor cycloheximide (2 μg/ml). [³H]-2-deoxyglucose uptake was measured. Transport rates are expressed as the mean ± SE of 4 replicates of a representative of 3 experiments. (The results shown in this figure were obtained with the assistance of a summer student, Crina Lador.)

Figure 5.9 Like DNP, induction of GLUT3 protein levels by rotenone is insensitive to cycloheximide. In contrast, the CoCl₂- and hypoxia-mediated increases in GLUT3 are blocked by the inhibitor

DIFFERENTIATED L6 CELLS WERE INCUBATED WITH DNP (0.5 mM), CoCl₂ (0.1 mM), INSULIN (100 nM), ROtenone (0.5 μg/ml) OR INCUBATED IN A HYPOTHETICAL CHAMBER (3% O₂), IN THE PRESENCE OF 2 μg/ml cycloheximide for 18 h. Total membranes were isolated and subjected to immunoblot analysis with polyclonal antibodies against GLUT1 (A) and GLUT3 (B). Representative gels of 4 replicates are shown. (The results shown in this figure were obtained with the assistance of a summer student, Crina Lador.)

DISCUSSION

In this chapter, the effect of chronic (18 h) treatment with DNP on GLUT3 and GLUT1 regulation in L6 myotubes was examined, and compared to the known effect of prolonged
insulin treatment. Prior studies have shown that, unlike insulin, the increase in glucose uptake mediated by DNP is insensitive to the protein synthesis inhibitor cycloheximide (23, 450), however the transporter(s) responsible for sustaining glucose transport was not investigated. The results presented in this chapter demonstrate that chronic DNP caused an elevation of GLUT1 mRNA and protein, suggesting that de novo biosynthesis of this isoform occurred, hence cycloheximide was able to block the DNP-stimulated elevation of GLUT1. On the other hand, prolonged DNP treatment elevated GLUT3 protein in L6 myotubes without changing the steady-state GLUT3 mRNA levels. Moreover, the uncoupler attained this increase independent of transcription or translation of new GLUT3 protein, but rather by a protein stabilization mechanism. The observation that DNP-stimulated glucose uptake and GLUT3 protein levels remained elevated during co-incubation with cycloheximide, suggests that GLUT3 makes a significant contribution to the DNP-stimulated component of glucose transport.

**GLUT1 may be Functionally Silent**

Interestingly, although chronic DNP treatment elevated both GLUT3 and GLUT1 protein levels, the elimination of the GLUT1 component by cycloheximide had minimal effect on DNP-stimulated glucose transport (Figure 5.5). Lack of correlation between GLUT1 levels and glucose transport has been noted in earlier reports. For example, glucose deprivation of L6 muscle cells augmented GLUT1 mRNA, GLUT1 protein and glucose transport, and the GLUT1 induction was cycloheximide-sensitive but not the glucose transport stimulation (450). The inhibitors of oxidative phosphorylation rotenone and azide also have discrepant effects on GLUT1 and glucose uptake in L6 muscle cells (23). A reasonable explanation of the results could be that the GLUT3 glucose transporter is responsible for the majority of the glucose transport stimulated by DNP irrespective of the elevated GLUT1 protein levels, i.e. that GLUT1 is silent during metabolic stress. The observation that the responses to rotenone and DNP are similar (Figures 5.9 and 5.8) is consistent with the possibility that GLUT3 predominates during the response to mitochondrial impediment. Indeed, the conclusion that GLUT1 is silent is not without precedent. Nishimura *et al.* observed that the catalytic efficiency of GLUT4 was far
greater than that of GLUT1 when both transporters were coexpressed in Xenopus oocytes (300). Moreover, Xia et al. have proposed that GLUT1 protein may normally exist in an inactive form in L6 muscle cells and that the GLUT3 and GLUT4 isoforms suffice to provide the cell with essential glucose transport activity (468). It is likely that GLUT3 serves a specific purpose in the metabolic adaptation of L6 muscle cells to stimuli which compromise energy supply such as disruption of the oxidative chain and glucose deprivation. However, whether GLUT3 serves the same purpose during hypoxic conditions is less clear because hypoxia and CoCl2 increased both GLUT1 and GLUT3 by similar protein synthesis-dependent mechanisms. Therefore the adaptive response to chronic metabolic demand appears to be very complex, engaging different cellular mechanisms depending on the nature of the metabolic insult.

**Unique Regulation of GLUT3**

This study reports for the first time the half-lives of GLUT3 and GLUT1 in L6 myotubes are about 15 h and 6 h, respectively. The mitochondrial uncoupler DNP slightly reduces the degradation of GLUT1 while it significantly prolongs the half-life of GLUT3. Effects at the level of GLUT translation cannot be ruled out because the rate of GLUT3 synthesis was not directly measured in this study. However, as an inhibitor of protein synthesis did not affect the elevation in GLUT3 mediated by DNP, this suggests that with DNP treatment, regulation at the level of protein stability is a more likely possibility.

Previous reports examining the effects of energy demand (glucose deprivation) and insulin on glucose transporter turnover have focused on GLUT4 and GLUT1 in 3T3-L1 cells (269, 351, 150). In those studies, insulin was shown to increase the rate of degradation of GLUT4 and GLUT1, while nutrient depletion led to increased stability of GLUT1 in 3T3-L1 adipocytes. Insulin slightly increases only GLUT3 and GLUT1 half-lives in L6 myotubes (Khayat and Klip, unpublished findings). Moreover, in adipocytes of fasting rats, the GLUT4 degradation rate was also increased (201). Therefore, the regulation of glucose transporter half-life is likely different in muscle and fat cells and is isoform-specific.
Elevations in GLUT1 mRNA and protein have been reported in response to chronic stimulation with energy stressors such as hypoxia and ischemia, and with insulin in the brain (266, 423), as well as glucose deprivation, hypoxia and mitochondrial uncouplers/inhibitors in muscle (23, 22, 450), fat (23, 130), and other tissues and cell lines (104. 364). For the most part, GLUT1 expression is controlled pre-translationally and the effects of insulin or other stimuli result from increased mRNA stability (268, 260, 20), transcription (450, 365), as well as by enhanced protein synthesis (351) and translation (400), and do not include mechanisms that stabilize glucose transporter proteins. The observations in this chapter of the effects of DNP, rotenone, CoCl₂ and hypoxia on GLUT1 expression are in good agreement with these previous studies.

Compared to GLUT1, relatively little is known about how the expression of GLUT3 is regulated. Physiological changes such as hypoxia-ischemia, chronic hypoglycemia and starvation have been shown to induce GLUT3 protein in the immature rat brain (434) and rat brain neurons (266), and increase GLUT3 mRNA in mouse brain (289). It has also been reported that chronic insulin increased GLUT3 protein and mRNA in L6 muscle cells (35, 398) and UMR 106-01 osteosarcoma cells (409). However, the cellular mechanisms responsible for mediating these changes in GLUT3 expression are unknown. We propose a unique mechanism of regulation whereby chronic mitochondrial disruption elicits GLUT3 protein stabilization, whereas chronic O₂ depletion increases GLUT3 biosynthesis.

Preliminary results reported here indicate that the lysosomal degradation of GLUT3 may be regulated by prolonged metabolic stress (Figure 5.7). In addition to the lysosome, the ubiquitin-proteosome pathway could also regulate GLUT protein turnover. Indeed, the ATP-dependence of proteosomal degradation (157) makes ubiquitination an intriguing possibility as a mechanism by which DNP elicits reduced GLUT3 degradation. An attempt was made to chemically block the ubiquitin-proteosome degradation pathway by the addition of a specific proteosomal inhibitor, lactacystin. Lactacystin interferes with proteasome activity by covalently inhibiting chymotrypsin- and trypsin-like activities of the β-subunit (101). Exposure of L6 myotubes to even the lowest recommended concentration of this compound (10 μM) lead to a
loss of viability, even within 4 h of treatment (Khayat and Klip, unpublished observations). Therefore the participation of the ubiquitin-proteasome pathway in the degradation of GLUT3 (or GLUT1), and its potential regulation by DNP has not yet been examined. New findings indicate that the sentrin-conjugating enzyme mUbc9 interacts with and covalently modifies GLUT4 and GLUT1 by conjugation with the ubiquitin-like substrate, sentrin (137). Although sentrin-conjugating enzyme regulates GLUT1 and GLUT4 protein levels in L6 myotubes, mUbc9 interaction with or regulation of the GLUT3 transporter isoform is absent (137). It is therefore not likely that alteration of this ubiquitin-like protein degradation pathway results in decreased GLUT3 protein degradation during prolonged metabolic stress in L6 cells.

CONCLUSIONS

The elevation of GLUT3 and GLUT1 protein mediated by mitochondrial uncoupling occurs by distinct mechanisms: the expression of the latter is governed by pre-translational processes, whereas elevation of GLUT3 is determined by increasing the half-life of the protein. In contrast to DNP, we found that hypoxia and insulin regulate both transporters by similar protein synthesis-dependent processes. These studies extend our understanding of the need for the expression of multiple glucose transport proteins in the same tissue for the regulation of glucose and energy homeostasis, and confirm that energy stressors and insulin continue to engage different glucose transport systems beyond the acute phase of glucose transport.
CHAPTER 6: OVERALL IMPLICATIONS AND FUTURE DIRECTIONS OF THE STUDY
OVERALL IMPLICATIONS OF THE STUDY

Uncovering the mechanisms of regulating glucose transport is essential for understanding physiological mechanisms that control glycemia and whole body glucose metabolism. This understanding is particularly important in diabetic patients who have a compromised responsiveness of glucose transport, leading to poor glycemic control and to a large number of diabetic complications. Due to its large tissue mass and rapid sensitivity to insulin, skeletal muscle tissue is the major site of insulin-mediated glucose disposal after a meal. These features make skeletal muscle a critical target in the therapy of insulin resistance in the diabetic state.

Whereas insulin stimulates muscle glucose metabolism and storage as glycogen in the resting state, during physical exercise or hypoxia, glucose catabolism is increased for rapid energy supply. Thus, skeletal muscle is also the principal site of glucose disposal and metabolism during metabolic demand. The increase in glucose uptake by skeletal muscle during hypoxia and contractions becomes of critical importance during diabetes where insulin resistance renders insulin an ineffective means of managing blood glucose levels. Consequently, exercise has long-been employed to control hyperglycemia associated with diabetes. It is therefore essential to understand the mechanisms employed by both insulin and hypoxia/exercise to regulate the rate of glucose transport into skeletal muscle cells so that cellular mediators may be targeted for therapeutic intervention.

The main scope of the work presented in this Thesis was to explore in detail the different intracellular mediators employed by insulin and the alternative activators of glucose uptake in muscle cells in culture. Although a role for the actin cytoskeleton in the insulin response (but not the alternative pathway) was certain, prior to these studies little was known about the metabolic consequences of insulin-dependent actin filament remodelling. The experimental studies for elucidating the insulin-dependent pathway focused on morphological analysis of the
spatial relationship between insulin-dependent actin remodelling, signal transduction, and GLUT4 mobilization (Chapter 3). Unlike insulin, very little is known about glucose transport regulation in the alternative pathway, except that this pathway does not utilize any of the mediators of the acute or chronic insulin-dependent glucose transport system. To explore insulin-independent glucose transport activation, experiments centered on identifying cellular mediators of the acute glucose transport response (Chapter 4), and focused on uncovering molecular mechanisms of upregulating glucose transporter expression during the chronic phase of glucose transport activation (Chapter 5).

THE ACTIN NETWORK AND INSULIN-DEPENDENT SIGNAL TRANSDUCTION TOWARDS GLUCOSE METABOLISM

The actin cytoskeleton participates in many cellular processes, including the maintenance of cell shape, coordinated cell movement, and intracellular traffic. To coordinate such a vast array of cellular functions, the actin cytoskeleton must be plastic, i.e. it must be able to adapt to rapidly changing conditions, such as those induced by polypeptide hormones. It is well established that insulin rapidly induces actin remodelling in a variety of cell types including L6 myotubes. However, a detailed analysis of the morphological changes in actin filaments brought about by insulin, and how they might be linked to the metabolic responses of the hormone was lacking. The results in Chapter 3 demonstrate that insulin provokes a rapid and marked aggregation of filamentous actin into structures that project from the dorsal surface of myotubes. These actin structures rapidly concentrate IRS-1, p85α, p110α, and Akt, and subsequently recruit GLUT4 organelles and the SNARE machinery required for the insertion of the GLUT4-containing vesicles into membrane ruffles.

The findings described in Chapter 3, in addition to other observations in the literature can be rationalized by the following hypothetical model for the participation of the actin-based
cytoskeleton in the exocytic transit of GLUT4 in L6 myotubes (Figure 6.1): In the basal state, the intracellular IRS-1, p85α, p110α, and aPKC polypeptides are tethered to cytoskeletal structures which may include F-actin (Figure 3.25), microtubules (313) or intermediate filaments (73). A portion of the GLUT4 (and GLUT1, but not GLUT3) vesicles in the LDM fraction are also tethered to the cytoskeleton in the basal state (Figure 3.26). On the other hand, the majority of cytosolic p110α is constitutively associated with the actin stress fibers running longitudinally along the myotube axis (Figure 3.11). Upon insulin binding to its receptor, IRS-1 becomes phosphorylated and activates PI3-K, which activates Rac [either by directly binding Rac or indirectly, e.g. via PI3-K lipid products (146, 226, 41)], leading to the formation of actin structures. Within minutes, actin filaments concentrate into projections at the dorsal surface of the muscle cell, where actin-bound p110α recruits the activated IRS-1-p85α complex (Figures 3.10, 3.11). By 10 min of insulin stimulation, a subpopulation of vesicles containing GLUT4 and VAMP2 are recruited to the newly formed actin structures (Figures 3.17 and 3.23), possibly via the partial tethering of GLUT4 to the cytoskeleton (Figure 3.26). The new actin structures bring GLUT4 vesicles within close proximity to their target membrane, and to the activated p85α-p110α PI3-K enzyme which phosphorylates lipid substrates within the GLUT4 endomembranes and/or the plasma membrane (Figure 3.12). The products of this reaction, PI 3,4,5-P3 and/or PI 3,4-P2 on either PM or vesicles gathered by the actin mesh, may then bind and activate the downstream effector Akt (via PDK1 and PDK2), leading to its appearance in the actin structures (Figure 3.14), and in GLUT4 vesicles (56, 236). The serine or threonine phosphorylation of a currently unidentified protein(s) (possibly SNARE proteins) triggers the insertion of GLUT4 vesicles into the plasma membrane, mediated by VAMP2 and t-SNAREs on the membrane ruffles.
In the basal state, p110α is tethered to actin filaments, while IRS-1 and p85α may be associated with other cytoskeletal elements. Occupation of the receptor by insulin leads to IRS-1 phosphorylation which recruits and activates PI3-K (p85α-p110α complex). Generation of PI-3,4,5-P₃ in intracellular membranes containing GLUT4 organelles and/or the plasma membrane induces local actin reorganization and simultaneously draws in Akt. The close proximity of Akt to GLUT4 organelles triggers the fusion of the organelles into ruffled regions of the plasma membrane in order to increase glucose flux into the myotube.

The hypothetical model, as presented, does not consider the possibility that in addition to compartmentalizing signalling molecules and GLUT4 organelles, actin microfilaments may serve as tracks for the physical movement of GLUT4 vesicles from the perinuclear region to the plasma membrane. The observation that complete depolymerization of actin filaments with CD or LB fully inhibited GLUT4 translocation, while inhibition of only the remodelling of actin filaments with swinholide-A reduced translocation by 62% (Figure 3.6) supports the notion that the actin network itself contributes to the full insulin response. Several recent reports have
implicated actin filaments in GLUT4 organelle traffic events in 3T3-L1 adipocytes (453, 317, 112). Therefore the participation of the actin network in GLUT4 movement in L6 myotubes is a distinct possibility which warrants further testing.

**Future Directions for the Study of the Cytoskeleton and Insulin-Dependent GLUT4 Translocation**

The model encompassing the results presented in Chapter 3 (Figure 6.1) opens the door to many attractive areas for future experimentation. To begin, a portion of intracellular GLUT4 and GLUT1 is insoluble in detergent, whereas the plasma membrane-associated proteins do not appear to be tethered to any cytoskeletal structures. Future studies could be aimed at uncovering cytoskeletal proteins that bind differently to isoforms of GLUTs, are compartment-specific, and whose binding is sensitive to insulin. This might lead to the identification of proteins that could eventually be tested as potential regulators of glucose transporter activity downstream of insulin. For these experiments, glucose transporters could be immunoprecipitated from intracellular and plasma membranes derived from control and insulin-treated cells. The copurification of the cytoskeletal proteins such as actin, ankyrin, spectrin, vinculin, etc. could be explored by immunoblotting using specific antibodies. The specificity of any detected interactions could be characterized by *in vitro* (*i.e.* GST pull-down) and *in vivo* (*i.e.* immunofluorescence localization) approaches.

The majority of the analysis in Chapter 3 focused on the GLUT4 protein because of its rapid responsiveness to insulin. However, in addition to GLUT4, GLUT1 and GLUT3 are also expressed in parental L6 myotubes (35). GLUT3 is largely on the cell surface and is less responsive to insulin (462). On the other hand, half of the GLUT1 is intracellular and is recruited to the cell surface by insulin (462). The intracellular GLUT1 segregates only partially with immunopurified GLUT4-containing intracellular compartments in L6 GLUT4myc cells (425), and, like GLUT4, was partially tethered to cytoskeletal structures (Figure 3.25). It would
therefore be interesting to perform an in-depth analysis of the consequences of actin filament remodelling on GLUT1 distribution and on its externalization in response to insulin. The GLUT1myc cell line currently being developed in the lab will be of great value for these studies, as it can be used to perform many of the experiments designed for the GLUT4myc cells in Chapter 3. These experiments will begin to reveal whether the formation of actin structures for propagating PI3-K-dependent signals is specific to GLUT4, or whether GLUT1 is also recruited by a similar mechanism.

Another interesting line of questioning will be the nature of the association of IRS-1, p85, and p110 with intracellular membranes (possibly the GLUT4-enriched compartment). This interaction likely involves linking through cytoskeletal elements because, in the LDM, these proteins were insoluble in Triton X-100 (Figure 3.26). It remains to be determined which cytoskeletal elements are sedimented with the detergent-insoluble material from LDM and whether/how these signalling proteins are directly tethered to those filamentous elements.

Although the morphological changes in actin and the cell surface elicited by insulin in L6 myotubes are striking, it will be important to determine whether insulin triggers similar phenomena in native skeletal muscle. The study described in Chapter 3 could be extended to explore whether the phenomena observed in L6 myotubes are a true reflection of the insulin response in primary myotubes or isolated muscle fibers derived from rodent skeletal muscle tissue. These proposed studies will also open the door to understanding the participation of cortical actin filaments in insulin-dependent muscle glucose transport in humans.

The observation that transfection of a dominant-negative mutant of Rac1 (Rac1-N17), but not the equivalent mutant of Rho, abrogates the formation of the cortical actin mesh and precludes GLUT4 incorporation into the cell surface in response to insulin was unprecedented (Figure 3.7). This finding could be built upon by focussing on the events linking the Rac
activation to actin remodelling. Several effectors of Rac members have been proposed to mediate actin remodelling, including the family of serine/threonine kinase p21-activated kinases (PAKs). Indeed, PAK2 is strongly activated by insulin in L6 myotubes (419). In addition to PAK, there is a cohort of Rac effectors that regulate the nucleation and branching at barbed ends of actin filaments and may be regulated by insulin. Actin-depolymerizing factor (ADF) and its related protein coflin (collectively called coflin) promote filament breakage as well as dissociation of actin monomers from the pointed end of filaments (57). Activated Rac increases association of PAK with Lim kinase-1 (LIMK-1) in fibroblasts, and LIMK-1 in turn phosphorylates and thereby inhibits coflin severing action (476). In addition to the PAK→cofilin axis, Rac also exerts its modulation of actin filament organization via the protein gelsolin, which severs and then caps the barbed end of actin-nucleating sites (390). Therefore, how PAK, LIMK-1, coflin and gelsolin become spatially and temporally coordinated in response to insulin, and whether these proteins contribute to insulin-induced actin remodelling and GLUT4 traffic will be an important area of future discoveries in this field.

The ongoing discoveries of several classes of unconventional myosins mediating movement of intracellular vesicles along actin tracks (274) point to the potential participation of myosin in actin-based GLUT4 vesicle traffic. Indeed, work is beginning to emerge showing that GLUT4-containing endomembranes are endowed with a new class of unconventional myosin, MM1b/Myr2 (43). Future studies investigating the participation of unconventional myosins in insulin-regulated GLUT4 translocation will hopefully shed light on the exact mechanism by which actin filaments participate in GLUT4 traffic events.

Finally, microtubules and intermediate filaments are known to interplay with the actin cytoskeleton, and many recent reports suggest that these other networks are involved in insulin-dependent GLUT4 traffic in fat cells in culture. For these reasons, it will be important to
examine the role of microtubules and intermediate filaments in insulin action, and to determine the relationship between the three cytoskeletal networks in L6 myotubes. As was performed for the studies described in Chapter 3, isoform-specific antibodies can be used to visualize the dynamics of tubulin or intermediate filament proteins upon insulin stimulation of L6 cells. The effect of chemically or molecularly interfering with the two filament systems on insulin-stimulated GLUT4 translocation (as has been reported in 3T3-L1 adipocytes), could reveal whether the microtubule and intermediate filament networks participate in similar events in L6 skeletal muscle cells.

The work described in Chapter 3 has already triggered an increased interest in the role of actin microfilaments in the mediation of insulin signalling and intracellular glucose transporter traffic in insulin-responsive cells. It is hoped that results from the many exciting potential future directions of the study will provide a comprehensive understanding of the role of the cytoskeleton in normal insulin action, and its defects in insulin resistance of diabetes.

**ACUTE REGULATION OF GLUCOSE UPTAKE BY METABOLIC DEMAND**

As mentioned above, it is of primary importance to understand the regulation of glucose transport in muscle, not only by insulin but by stimuli that increase energy demand. Understanding the mechanism of action of hypoxia and exercise on the glucose transport system is important not only to define the physiological function of skeletal muscle during metabolic stress, but also because the knowledge gained may be useful in defining more selective methods for the management of glycemia in cases of insulin resistance where insulin cannot regulate glucose transport effectively. The mitochondrial uncoupler, DNP is a convenient tool to mimic hypoxia and study the mechanism of the stimulation of glucose transport in hypoxia-like conditions in muscle cells in culture. For those reasons, in Chapter 4 it was attempted to first
clarify that insulin and DNP utilize independent pathways to elevate glucose transport, and, secondly, to elucidate mediators of the DNP response. The results in Chapter 4 and previous findings in L6 myotubes reveal that DNP and insulin increase glucose uptake in an additive manner (Figure 4.1), and that DNP does not engage any insulin signalling molecules (Figure 4.2) or the actin network (418) to mediate the glucose transport response. The findings also demonstrate that DNP-stimulated glucose uptake largely depends on cytosolic Ca\(^{2+}\) (Figure 4.3) and Ca\(^{2+}\)-sensitive PKCs (Figures 4.5 and 4.6). On the other hand, the fuel sensor AMPK does not appear to participate in the metabolic demand pathway in L6 (316), although the enzyme is activated by DNP (Figure 4.12). Taken together, the results presented in Figure 4 raise the possibility that 3 types of signals cooperate to bring about the DNP effect on glucose uptake: cPKC activation, a secondary effect of Ca\(^{2+}\), and a Ca\(^{2+}\)-independent signal. This concept is illustrated in Figure 6.2.

**Figure 6.2 Ca\(^{2+}\) and cPKC-dependent components of DNP-stimulated glucose transport**

The pie graph represents 100% of the glucose transport stimulated by DNP. The hatched and dotted regions represent the Ca\(^{2+}\)-dependent component (80%) of this activation. Within this Ca\(^{2+}\)-dependent element, we postulate that 60% of the DNP response (dotted region) is mediated by cPKC's that are sensitive to intracellular Ca\(^{2+}\) levels (likely PKC-β). The remaining Ca\(^{2+}\)-independent component (~20%, white region) involves unknown mediators.

**Future Directions for Studying the Acute Metabolic Demand Glucose Transport System**

The findings presented in Chapter 4 have opened the door to a number of potentially exciting avenues for research on this subject to continue. For one, it has not yet been directly demonstrated that DNP, indeed, increases [Ca\(^{2+}\)], although Ca\(^{2+}\) chelation prevented DNP-stimulated glucose uptake (Figure 4.3). It would therefore be useful to characterize the Ca\(^{2+}\) transients generated by DNP in L6 cells. This could be accomplished by loading myotubes with
the Ca\(^{2+}\)-sensitive fluorophore, INDO1-AM and monitoring DNP-sensitive changes in INDO1 fluorescence emission. Because DNP is a chromophore itself, the interference of the compound with the excitation or emission of INDO1 may pose some challenges. Other mitochondrial inhibitors such as rotenone or the uncoupler, CCCP could substitute for DNP, if necessary. These proposed experiments would ascertain the time course and magnitude of Ca\(^{2+}\) changes induced by DNP and would allow for the correlation of these parameters with the glucose transport data.

It will also be important to determine whether the observations made here in L6 skeletal muscle cells reflect the physiological response to exercise or hypoxia in skeletal muscle in vivo or in vitro. Although the participation of Ca\(^{2+}\) and PKC in the exercise pathway in muscle has been explored in the past, those previous studies were performed before the reagents employed in Chapter 4 were discovered. Therefore, it would be useful to revisit the involvement of Ca\(^{2+}\) and cPKC in contraction-stimulated glucose uptake in skeletal muscle using BAPTA-AM and BIM-1, respectively.

The additivity of insulin- and DNP-stimulated glucose uptake (Figure 4.1) indicates that L6 myotubes possess different pools of GLUT4 that are recruited to the plasma membrane by the two stimuli, reflecting the situation in skeletal muscle. Therefore L6 cells could be used as a model system for future studies exploring the biology of exercise- and insulin-stimulated GLUT4 compartments, and the vesicular machinery (SNARE proteins) that regulates the redistribution of these organelles in response to energy demand in skeletal muscle.

Surprisingly, the results in Chapter 5 reveal that oxygen depletion (hypoxia or CoCl\(_2\)) and mitochondrial inhibition (DNP, rotenone) do not behave similarly in the chronic glucose transport response in L6 myotubes, although all treatments significantly elevate glucose uptake. It would therefore be worthwhile to compare the effect of acute exposure of L6 cells to hypoxia,
CoCl$_2$ and rotenone to the response to DNP to determine whether all types of metabolic stress employ the same mediators (e.g. Ca$^{2+}$ and cPKC). Like the chronic glucose transport phase, these proposed studies could uncover novel differences in the response to decreased ATP production versus decreased O$_2$ availability in the mitochondria.

Another major unresolved issue is whether other signalling molecules, in addition to PKC, are responsible for mediating DNP's effect on glucose transport. A direct link between ATP concentration and glucose transport regulation has not been established to date. However, it is likely that the rapid drop in ATP levels caused by DNP may signal the activation of other molecules participating in the stimulation of the glucose uptake system, although the participation of AMPK is not likely. Several other potential mediators have been proposed (see Background section) that are worthy of testing in DNP-treated L6 muscle cells. These include NO, bradykinin, adenosine, and CaMKII. It would also be potentially interesting to test whether DNP, in addition to translocating GLUTs, also activates the transporters in the plasma membrane. Recent reports indicate that the enzyme p38MAPK may be involved in the activation of glucose transporters in the insulin-dependent pathway (see Background). Indeed DNP and exercise elicit an increase in the activation (phosphorylation) of p38MAPK in L6 muscle cells (398), and skeletal muscle (373), respectively. To determine whether DNP-stimulated glucose uptake activation depends on p38MAPK, molecular or pharmacological approaches of interfering with p38MAPK could be tested, as has already been extensively explored for the insulin-dependent pathway.

Finally, a physiological mechanism of regulating mitochondrial respiration not yet discussed in this Thesis is through the action of a family of mitochondrial uncoupling proteins (UCP), of which 4 isoforms have been identified [reviewed in (173)]. Classically, UCPs are thought to increase thermogenesis due to the high levels of UCP in brown adipocyte tissue.
UCP2 and UCP3 isoforms are highly expressed in skeletal muscle (265), and there have been reports that contractions increase UCP2 and UCP3 expression in muscle by an AMPK-dependent manner (488, 319). It has also been recently reported that overexpression of UCP3 increases glucose uptake and cell surface GLUT4 content in L6 myotubes, but not 3T3-L1 adipocytes (167). Therefore, it would be interesting to examine if the rapid turning on and off of UCP activity in muscle is a mechanism of acutely regulating glucose metabolism.

Clearly, it is of great interest and potential use to determine which signals trigger glucose transporter mobilization and activation in response to energy stressors. And it would be especially useful to selectively capitalize on this pathway to counteract insulin resistance without inhibiting aerobic metabolism.

REGULATION OF GLUT EXPRESSION BY PROTEIN DEGRADATION
The regulation of glucose transporter expression is a critical feature of cellular and, in turn, whole body glucose homeostasis, as changes in specific glucose transporter expression can lead to profound alterations in cellular physiology, including insulin resistance and ischemia. To comprehend the consequence of aberrant GLUT expression during such pathological states requires a thorough understanding of the numerous processes that might affect glucose transporter protein levels in the cell and their regulation by diverse stimuli. Before the study presented in Chapter 5 was published, little was known about the regulation of GLUT3: now it is known that GLUT3 is recruited to the cell surface by insulin but not by DNP (418), and that although prolonged exposure to insulin increases its synthesis (Figure 5.4), chronic exposure to DNP increases the half-life of the GLUT3 protein (Figure 5.6). Furthermore, GLUT1 biosynthesis is activated by both stimuli, although GLUT1 protein is likely not functional during the adaptive response to prolonged exposure to DNP (Figures 5.4 and 5.5). A surprising finding
was that chronic inhibition of mitochondrial O₂ uptake by exposing cells to CoCl₂ or hypoxia also increases GLUT3 (and GLUT1) protein levels, but by biosynthetic, rather than, post-translational means (Figure 5.8). On the other hand, the mitochondrial inhibitor, rotenone, increases GLUT1 and GLUT3 expression by similar processes as DNP. The molecular mechanisms by which these energy stressors and insulin chronically control glucose transporter expression and glucose uptake are summarized in Table 6.1. In all, these findings have begun to piece together the different roads that lead to altered glucose homeostasis during chronic exposure to insulin and metabolic demand.

Table 6.1 Summary of the major mechanisms of glucose transporter regulation by the stimuli studied in Chapter 5

<table>
<thead>
<tr>
<th></th>
<th>Insulin GLUT1</th>
<th>Insulin GLUT3</th>
<th>DNP GLUT1</th>
<th>DNP GLUT3</th>
<th>Hypoxia GLUT1</th>
<th>Hypoxia GLUT3</th>
</tr>
</thead>
<tbody>
<tr>
<td>steady-state mRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steady-state Protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+CHX (protein)</td>
<td>▼ to basal</td>
<td>▼ to basal</td>
<td>▼ to basal</td>
<td>▼</td>
<td>▼ to basal</td>
<td>▼ to basal</td>
</tr>
<tr>
<td>Major Mechanism</td>
<td>biosynthesis</td>
<td>biosynthesis</td>
<td>biosynthesis</td>
<td>▼ protein degradation</td>
<td>biosynthesis</td>
<td>biosynthesis</td>
</tr>
</tbody>
</table>

+CHX, effect of cycloheximide on protein content; ▼, increase; ▼, decrease; —, no change; ND, not determined.

Future Directions for Understanding the Chronic Metabolic Demand Glucose Transport System

From the studies presented in Chapter 5, many questions arise which warrant further examination. The first is, do the acute signals elucidated in Chapter 4 also contribute to enhanced GLUT1 and GLUT3 expression in the chronic response? That is, in addition to rapidly signalling the translocation of glucose transporters to the plasma membrane, perhaps Ca²⁺ and cPKC contribute to the increased synthesis and stability of GLUT1 and GLUT3 proteins (respectively) during prolonged exposure to DNP. To test this possibility, the strategies used to interfere with Ca²⁺ and cPKC signalling in Chapter 4 could be applied to chronic (18 h) DNP treatments in order to examine their effects on steady state GLUT levels and glucose transport. If 18 h of BAPTA-AM or BIM-I proves too toxic to the myotubes, 4 h of DNP
stimulation could be used since at this time point GLUT1 and GLUT3 protein levels are already significantly elevated (Figure 5.4).

Although the results in Chapter 5 strongly suggest that GLUT3 protein predominates in the adaptive response to long-term DNP exposure, the experiments presented examined steady-state GLUT3 (and GLUT1) protein levels in total membranes (LDM and PM) of L6 myotubes. It is not certain that the increased glucose transport observed in the chronic phase reflects the elevated content of these glucose transporters in the plasma membrane per se. These studies would therefore be strengthened if future work focused on comparing the effect of DNP ± cycloheximide on GLUT3 and GLUT1 content in subcellular membrane fractions (PM and LDM) in L6 myotubes.

The striking contrast between chronic hypoxia and mitochondrial inhibition on GLUT expression (Figure 5.8) paves the way for exciting future experiments aimed at discerning how/why the mechanisms are distinct. For these studies the effect of chronically exposing L6 myotubes to hypoxia or CoCl$_2$ on GLUT1 and GLUT3 mRNA levels, protein synthesis, and, perhaps protein translation could be examined. These molecular techniques have all already been established in our laboratory for studies examining the chronic effects of insulin on these parameters in L6 myotubes.

Finally, controlled protein degradation is a new and important area in cell biology, and little is known about the possible participation of insulin or other activators of glucose transport in this phenomenon. Protein degradation occurs primarily by two mechanisms: ubiquitin tagging for proteasome degradation, and delivery to the lysosome for degradation of endocytosed components. Recent studies indicate that membrane proteins can be degraded by the ATP-dependent proteasome pathway, but whether glucose transporters follow this route, and if and how this is regulated, is not known. A focus of future studies in this area could be testing
the hypothesis that signals from continuous exposure to DNP may have an impact on slowing the degradation of GLUT3, and to compare and contrast these findings with GLUT1 and GLUT4 regulation, and with the effects of prolonged insulin stimulation. Preliminary results using the lysosomal inhibitor E64d (Figure 5.7) suggest that the routing of GLUT1 or GLUT3 to the lysosome is a potential mechanism which could be explored further. These results could be corroborated by monitoring the effect of interfering with lysosomal acidification on the expression of GLUT1 and GLUT3 proteins. This approach could be complemented by detection of GLUT immunodetectable peptides in the lysosome by double-labelling immunocytochemistry, as well as by biochemical isolation of lysosomes followed by immunodetection of GLUT proteins within the lysosomal fractions.

It is equally possible that sustained exposure to DNP may diminish the ubiquitin signal on GLUT3. To test this possibility, inhibition of various peptidases of the 20S proteasome pathway with lactacystin was attempted in Chapter 5, but proved to be too toxic to L6 myotubes. Ubiquitin inhibition can also be achieved by transfecting L6 cells with an inactive (E1 enzyme) form of ubiquitin. It could also be explored whether any of the GLUTs are susceptible to ubiquitination, and if DNP or insulin alter the efficiency or rate of this process. Ubiquitination could be measured by immunoprecipitation of each GLUT followed by ubiquitin detection by immunoblotting. Conversely, all ubiquitinated proteins could be immunoprecipitated, and the presence of specific GLUT proteins could be probed for by immunoblotting with their specific antibodies. If the ubiquitin signal on GLUT proteins is not detectable by these approaches, an alternative strategy could be transfection of L6 cells with HA-tagged ubiquitin to facilitate immunoblotting or immunoprecipitation of ubiquitin via its tag epitope.
The study of GLUT degradation is novel and the proposed future studies will reveal the molecular mechanisms involved. It will be especially attractive to direct these studies to the future design of approaches to increase GLUT stability, which could be useful in the treatment of hyperglycemia \textit{in vivo}.

**FINAL SUMMARY**

The acute and chronic effects of insulin and DNP on glucose transport in muscle cells demonstrate the importance of glucose transport in maintaining glycemia and ATP production, respectively, and indicate that signalling pathways regulating glucose transporter translocation and expression can be accessed by factors other than insulin. As summarized in Figure 6.3, the working hypothesis is that these two pathways of glucose transport regulation involve differences between insulin and DNP in reorganizing the cytoskeleton, in summoning different signalling proteins, and in controlling glucose transporter biosynthesis and degradation. The model portrays a scheme whereby two different stimuli trigger two entirely different cellular mechanisms to produce the same endpoint. That is, both insulin and metabolic stress increase the number of glucose transporters in the plasma membrane, whether it is by accelerating the delivery of GLUT4 to the cell surface, or by increasing GLUT1 or GLUT3 protein levels. However, that fate of the glucose molecule transported into the cell in the hormonal versus metabolic response is different. Whereas insulin activates pathways which lead to glucose storage in the form of glycogen, the glucose which enters the cell during energy demand is destined for glycolytic ATP production. It is therefore tempting to speculate that the elements of the two glucose transport pathways depicted in Figure 6.3 are themselves compartmentalized into metabolons which also contain the enzymes required to transform the transported glucose into its impending metabolic substrate. Indeed, the evidence for metabolons of glycolytic enzymes is well-documented [reviewed in (376)].
The findings of Chapters 3-5 reveal distinct mediators of the two pathways for activating glucose transport in muscle cells. On the left side, the mechanism stimulated by insulin involves the PI3-K-dependent remodelling of actin filaments into structures which bring insulin signalling molecules into close proximity with the insulin-sensitive intracellular GLUT4 pool. Prolonged exposure to insulin triggers a mitogenic response which upregulates the biosynthesis of GLUT1 and GLUT3 (398). The goal of the insulin-dependent pathway is glucose storage as glycogen. On the right side, the pathway activated by DNP involves the Ca\(^2\)+ and cPKC-dependent recruitment of the 'exercise'-sensitive GLUT4 pool to the plasma membrane by a currently unknown mechanism. In parallel, AMPK is rapidly activated by DNP but likely does not feed in to the glucose transport system. Chronic DNP or rotenone treatment increases the cellular content of GLUT3 and GLUT1 by increasing the stability and biosynthesis of the proteins, respectively. Longterm exposure to hypoxia (physiological or chemical) increases GLUT1 and GLUT3 biosynthesis, without affecting the degradation of these proteins. Unlike insulin, the goal of the alternative pathway is glucose metabolism into ATP.
In all, the findings presented in this Thesis have enhanced our understanding of the diverse mechanisms of controlling muscle glucose uptake. The ultimate goal of the proposed future studies would be to understand how to individually control each transporter to reduce insulin resistance and improve glycemic control in humans with diabetes.
REFERENCES


118. Foran, PG, Fletcher, LM, Oatey, PB, Mohammed, N, Dolly, JO and Tavare, JM. 1999. Protein kinase B stimulates the translocation of GLUT4 but not GLUT1 or transferrin receptors in 3T3-L1 adipocytes by a pathway involving SNAP-23, synaptobrevin-2, and/or cellubrevin. J. Biol. Chem., 274: 28087-28095.


195


204


318. Pedersen, O, Bak, JF. Andersen, PH. Lund, S. Moller, DE. Flier, JS and Kahn, BB. 1990. Evidence against altered expression of GLUT1 or GLUT4 in skeletal muscle of patients with obesity or NIDDM. Diabetes, 39: 865-870.


COPYRIGHT RELEASE AUTHORIZATIONS
Dear Editor of Canadian Journal of Applied Physiology:

I am completing a doctoral Thesis at the University of Toronto entitled, "Multiple Mechanisms of Regulating Glucose Transporters and Glucose Transport in Skeletal Muscle Cells." I would like permission to allow inclusion of the following material in the Thesis and permission for the National Library of Canada to make use of the Thesis (i.e. to reproduce, loan, distribute, or sell copies of the Thesis by any means and in any form or format).

These rights will in no way restrict publication of the material in any other form by you or by others authorized by you. The excerpt to be reprinted is Figure 1 which is in press in Khayat, Patel and Klip, 2001, Can. J. Appl. Physiol.

If these arrangements meet with your approval, please sign this letter where indicated below and return it to me by fax or mail (below) before August 15th, 2001. Thank you for your assistance in this matter.

Yours sincerely,

Zayna A. Khayat
Cell Biology Programme
Hospital for Sick Children, rm 5001 McMaster Building
Toronto, Ontario, Canada M5G 1X8
Phone: 416-813-6612
Fax: 416-813-5028

PERMISSION GRANTED FOR THE USE REQUESTED ABOVE:

Signature: [Signature]
Print name: [Name]
Editor: [CJAP]
Date: July 30/01
Dear Mr/Ms Khayat

DIABETES ANNUAL, Vol 12, 1999, pp 111-132, Foster et al, Figure 4 only

As per your letter dated 27th July 2001, we hereby grant you permission to reprint the aforementioned material at no charge in your thesis subject to the following conditions:

1. If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies.

2. Suitable acknowledgment to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Journal title, Volume number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier Science".

3. Reproduction of this material is confined to the purpose for which permission is hereby given.

4. This permission is granted for non-exclusive world English rights only. For other languages please reapply separately for each one required. Permission excludes use in an electronic form. Should you have a specific electronic project in mind please reapply for permission.

5. This includes permission for the National Library of Canada to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

Yours sincerely

Frances Rathwell (Mrs)
Global Rights Manager

The processing of permission requests for all Elsevier Science (including Pergamon imprint) journals has been centralised in Oxford, UK. Your future requests will be handled more quickly if you write directly to: Subsidiary Rights Department, Elsevier Science, PO Box 800, Oxford OX5 1DX, UK.
Fax: 44-1865 853333; e-mail: permissions@elsevier.co.uk
Dear Editor of the Journal of Cell Science:

I am completing a doctoral Thesis at the University of Toronto entitled, "Multiple Mechanisms of Regulating Glucose Transporters and Glucose Transport in Skeletal Muscle Cells." I would like permission to allow inclusion of the following material in the Thesis and permission for the National Library of Canada to make use of the Thesis (i.e., to reproduce, loan, distribute, or sell copies of the Thesis by any means and in any form or format).

These rights will in no way restrict publication of the material in any other form by you or by others authorized by you. The excerpts to be reprinted are all figures which appear in Khayat et al, 2000, J. Cell Sci. 113: 279-290.

If these arrangements meet with your approval, please sign this letter where indicated below and return it to me by fax or mail (below) before August 15th, 2001. Thank you for your assistance in this matter.

Yours sincerely,

Zayna A. Khayat
Cell Biology Programme
Hospital for Sick Children, rm 5001 McMaster Building
Toronto, Ontario, Canada M5G 1X8
Phone: 416-813-6612
Fax: 416-813-5028

PERMISSION GRANTED FOR THE USE REQUESTED ABOVE:

Signature __________________________ Print name __________________________ Date ______________

PERMISSION GRANTED
PLEASE CREDIT JOURNAL REFERENCE AND COMPANY OF BILOGISTS LTD.

COMPANY SECRETARY
July 27, 2001

Dear Editor of the Journal of Clinical Investigation:

I am completing a doctoral Thesis at the University of Toronto entitled, "Multiple Mechanisms of Regulating Glucose Transporters and Glucose Transport in Skeletal Muscle Cells." I would like permission to allow inclusion of the following material in the Thesis and permission for the National Library of Canada to make use of the Thesis (i.e. to reproduce, loan, distribute, or sell copies of the Thesis by any means and in any form or format).

These rights will in no way restrict publication of the material in any other form by you or by others authorized by you. The excerpt to be reprinted is Figures 1 to 5 which are currently in press in Tong et al, 2001, J. Clin. Invest., 108 (3).

If these arrangements meet with your approval, please sign this letter where indicated below and return it to me by fax or mail (below) before August 15th, 2001. Thank you for your assistance in this matter.

Yours sincerely,

Zayna A. Khayat
Cell Biology Programme
Hospital for Sick Children, rm 5001 McMaster Building
Toronto, Ontario, Canada M5G 1X8
Phone: 416-813-6612
Fax: 416-813-5028

PERMISSION GRANTED FOR THE USE REQUESTED ABOVE:

Signature: [Signature]
Print name: [Print name]
Date: 8/15/01

[Apologies for the Madness!]

Zayna A. Khayat
Cell Biology Programme
Hospital for Sick Children, rm 5001 McMaster Building
Toronto, Ontario, Canada M5G 1X8
Phone: 416-813-6612
Fax: 416-813-5028
Dear Editor of the American Journal of Physiology Cell Physiology:

I am completing a doctoral Thesis at the University of Toronto entitled, "Mechanisms of Regulating Glucose Transporters and Glucose Transport in Skeletal Muscle Cells." I would like permission to allow inclusion of the following material in the Thesis and permission for the National Library of Canada to make use of the Thesis (i.e. to reproduce, loan, distribute, or sell copies of the Thesis by any means and in any form or format).

These rights will in no way restrict publication of the material in any other form by you or by others authorized by you. The excerpts to be reprinted are Figures 1-10 as they appear in Khayat et al., 1998, Am. J. Physiol. Cell Physiol. 275: C1487-C1497.

If these arrangements meet with your approval, please sign this letter where indicated below and return it to me by fax or mail (below) before August 5, 2001. Thank you for your assistance in this matter.

Yours sincerely,

Zayna A. Khayat
Cell Biology Programme
Hospital for Sick Children, rm 5001 McMaster Building
Toronto, Ontario, Canada M5G 1X8
Phone: 416-813-6612
Fax: 416-813-5028

PERMISSION GRANTED FOR THE USE REQUESTED ABOVE:

Signature

Print name

Date

THE AMERICAN PHYSIOLOGICAL SOCIETY
9650 Rockville Pike - Bethesda, MD 20814

Permission is granted for use of the material specified above provided the publication is credited as the source.

Date
Publ Mgr & Exec Editor

A health care, teaching and research centre dedicated exclusively to children; affiliated with the University of Toronto
Dear Dr. Khayat,

Thank you for your request to use material from an Academic Press publication.

It is now the policy of Academic Press that authors need not obtain permission in the following cases: (1) to use their original figures or tables in their future works; (2) to make copies of their papers for their classroom teaching; and (3) to include their papers as part of their dissertations. Of course, citation to the original source should be included.

Sincerely,

Stephen Sims
Publishing Services Editor
Academic Press
(619) 699-6813