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EXERCISE-INDUCED PROTECTION AGAINST INSULIN RESISTANCE
AND TYPE II DIABETES:

THE ROLE OF CALCIUM/CALMODULIN DEPENDENT PROTEIN KINASE
IN GLUCOSE TRANSPORTER-4 EXPRESSION

University of Cape Town

James Antony Harris Smith

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Thesis presented for the Degree of
DOCTOR OF PHILOSOPHY in EXERCISE SCIENCE

In the department of Human Biology

UNIVERSITY OF CAPE TOWN

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DECLARATION

I, James Antony Harris Smith, hereby declare that the work on which this dissertation is based is my original work (apart from the normal guidance from my supervisor and except where acknowledgments indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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PUBLICATIONS ASSOCIATED WITH THIS THESIS

Full Papers

J. A. H. Smith, A. Jennings and E. O. Ojuka. CaMK activation during exercise is required for histone hyper-acetylation and MEF2A binding at the MEF2 site on the *Glut4* gene. In Review, first submitted November, 2007.

E. Mukwevho, T.A. Kohn, D.M. Lang, E. Nyatia, **J. A. H. Smith** and E.O. Ojuka. Caffeine induces hyper-acetylation of histones at the MEF2 site on the *Glut4* promoter and increases MEF2A binding to the site via a CaMK-dependent mechanism. *Am J Physiol Endocrinol Metab* *In press*. 2008.

J. A. H. Smith, M. Collins, L. Grobler, C. Magee and E. O. Ojuka. Exercise and CaMK activation both increase the binding of MEF2A to the *Glut4* promoter in skeletal muscle *in vivo*. *Am J Physiol Endocrinol Metab* 292: E413-E420, 2007.

Professional presentations

MRC Research Day, Cape Town, South Africa, October 2007. Free communication: "The role of CaMK in Glucose transporter-4 expression".

35th meeting of the Physiological Society of Southern Africa, Johannesburg, South Africa, September 2007. Free communication: "CaMK is required for the expression of the skeletal muscle glucose transporter, GLUT4, in response to exercise".

International Diabetes Federation Conference, Cape Town, South Africa, Dec 2006. Poster session: "Exercise-induced GLUT4 expression is attenuated by KN93 in rat skeletal muscle".

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33rd meeting of the Physiological Society of Southern Africa, Cape Town, South Africa, Sept 2005. Free communication: “The role of CaMK in exercise-induced GLUT4 biogenesis”.

Experimental Biology 2005 and XXXV International Congress of Physiological Sciences, San Diego, CA, April 2005. Poster session: “Exercise-induced GLUT4 biogenesis involves CaMK and HDAC5”.

Experimental Biology Group Meeting. Cape Town, South Africa, May 2004, Free communication: “GLUT4 regulation in skeletal muscle involves CaMK and HDAC5”.

XIXth South African Society of Biochemistry and Molecular Biology Conference, Stellenbosch, South Africa, July 2004. Poster session: “GLUT4 regulation in skeletal muscle involves Ca²⁺/Calmodulin-dependent protein kinase and Histone Deacetylase 5”.

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LIST OF ABBREVIATIONS

AcH3 - acetyl histone H3

AICAR - 5-aminoimidazole-4-carboxyamide ribonucleoside

AMPK - adenosine monophosphate (AMP) activated protein kinase

ATF-1 - activating transcription factor-1

ATP - adenosine 5'-triphosphate

AVP - arg-vasopressin

bp - base pairs

BSA - bovine serum albumin

c/EBP β - CAAT- enhancer binding protein

C terminus - carboxyl terminus

C2C12 - mouse muscle cell line

CA - constitutively active

Ca²⁺ - calcium

CaCl₂ - calcium chloride

CaM - calmodulin

CaMK - Ca²⁺/calmodulin dependent protein kinase

CAT - chloramphenicol acetyltransferase

CBP - CREB-binding protein

cDNA - complementary DNA

ChIP - chromatin immunoprecipitation

CMV - cytomegalovirus

CON - control

COS 7 - monkey kidney cell line

CREB - cAMP response element binding protein

DMEM - dulbecco's modified eagles medium

DN - dominant negative

DNA - deoxyribonucleic acid

dNTP - deoxyribonucleotide triphosphate

DTT - dithiothreitol

E. coli - Escherichia coli

EDL - extensor digitorum longus

EDTA - ethylene diamine tetra-acetic acid

EGTA - ethylene glycol tetra-acetic acid

EMSA - electrophoretic mobility shift assays

EX + KN93 - rats that were injected with KN93 prior to completing a 5x17 minute swim

EX - rats that were injected with saline prior to completing a 5x17 minute swim

FBS - foetal bovine serum

GAPDH - glyceraldehyde 3-phosphate dehydrogenase

GEF - GLUT4 enhancer factor

GFP - green fluorescent protein

GLUT1/4 - glucose transporter-1/4 protein or mRNA

Glut4 - promoter or gene coding for GLUT4

GTE - solution containing glucose, Tris-HC and EDTA

HAT - histone acetyl transferase

HCl - hydrochloric acid

HDAC - histone deacetylase

HEK - human embryonic kidney

HEPES - 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid

HES - buffer containing HEPES, EDTA and Sucrose

HRP - horseradish peroxidase

HS - horse serum

IGF-1 - insulin like growth factor-1

IgG - immunoglobulin G

In vitro - (Latin: within the glass) refers to the technique of performing an experiment in a controlled environment outside of a living organism

In vivo - (Latin: within the living) refers to the technique of performing an experiment inside an organism

Input - sample used for ChIP assay prior to immunoprecipitation step

i.p. - intraperitoneal

IR - insulin receptor

IRS 1/2 - insulin receptor substrate 1/2

Kan - kanamycin resistant gene

KLF-15 - kruppel like factor-15

KN62 - CaMK II inhibitor

KN93 - CaMK II inhibitor

KN92 - inactive analogue of KN93

KOH - potassium hydroxide

L6 - rat muscle cell line

L-broth - luria broth

Leu - leucine

Lys - Lysine

MAPK - mitogen activated protein kinases

MCK - muscle creatine kinase

MEF2 - myocyte enhancer factor 2

MgCl₂ - magnesium chloride

MKK - mitogen-activated protein kinase kinase

MMLV - Moloney Murine Leukemia Virus

MOPS - 1% 3-(N-morpholino) propanesulfonic acid

MP - non fat milk powder

mRNA - messenger ribonucleic acid

N terminus - amino terminus

Na₃VO₄ - sodium orthovanadate

Na₄P₂O₇ - sodium pyrophosphate

NaCl - sodium chloride

NaF - sodium fluoride

NaHCO₃ - sodium bicarbonate

NaOH - sodium hydroxide

NFAT - nuclear factor of activated T-cell

NP-40 - nonidet P-40

Ori - origin of replication

³²P - phosphate radioisotope

pAd-CaMK IV - adenoviral vectors containing a human CaMK IV gene

pAd-CON - adenoviral vector that does not contain an inserted recombinant gene

PAGE - polyacrylamide gel electrophoresis

PBST - phosphate buffered saline (PBS) containing 0.1% Tween 20

PCAF - p300/CBP-associated factor

PCR - polymerase chain reaction

PDK - phosphoinositol dependent kinase

PGC-1α - peroxisome proliferator-activated receptor gamma coactivator-1 alpha

PI3 kinase - phosphatidylinositol 3-kinase

PKB - protein kinase B

PMSF - phenylmethanesulphonyl fluoride

PVDF - polyvinylidene difluoride

qRT-PCR - quantitative real time polymerase chain reaction

RS12 - ribosomal protein S12

RNA - ribonucleic acid

SDS - sodium dodecyl sulphate

Ser - serine

SRF - serum response factor

STZ - streptozotocin

T200A - mutation of the threonine residue at amino acid position 200 to alanine

TBST - Tris buffered saline (TBS) containing 0.1% Tween 20

TE - buffer containing Tris-Cl and EDTA

Thr - threonine

TRE - thyroid hormone receptor

TR α 1 - thyroid receptor α 1

VO₂ peak - peak oxygen consumption

WHO - world health organisation

WT - wild type

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ABSTRACT

Regular exercise protects individuals against developing insulin resistance and type II diabetes. This effect of exercise does not appear to be due to an improvement in the insulin signalling pathway but instead due to an increase in the content of the insulin-regulatable glucose transporter (GLUT4) in skeletal muscle (84). Understanding the mechanisms by which exercise increases GLUT4 levels in skeletal muscle may reveal targets for pharmaceuticals to treat insulin resistance and type II diabetes. Although *in vitro* binding assays have shown that GLUT4 expression during exercise is mediated by the binding of myocyte enhancer factor-2A (MEF2A) to its *cis*-element on the *Glut4* promoter (122), this has not been demonstrated *in vivo*. Moreover, the mechanisms by which exercise increases MEF2A binding to the *Glut4* promoter have not been fully characterised. There is evidence from cell culture that Ca²⁺/Calmodulin dependent protein kinase (CaMK) plays a role in skeletal muscle GLUT4 expression (150) and CaMK has been known to enhance transcription of MEF2 regulated genes by influencing the acetylation of histones in gene promoters (181).

The objectives this thesis therefore were (a) to evaluate the effects of exercise on the *in vivo* binding of MEF2A to its *cis*-element in the *Glut4* promoter; the *in vivo* acetylation of histones at this site; and the expression of GLUT4 mRNA and protein, in skeletal muscle, (b) to investigate whether CaMK activity was necessary for these exercise- induced responses, and (c) to determine whether CaMK activity was sufficient to increase GLUT4 expression and MEF2A binding to the *Glut4* promoter. The following hypotheses were therefore investigated:

- (a) A bout of exercise will increase the binding of MEF2A to its binding site on the *Glut4* promoter and increase the acetylation of Histone H3 at this site, *in vivo*.
- (b) Inhibition of CaMK activity in skeletal muscle by KN93 will abolish the increases in GLUT4 mRNA and protein levels, MEF2A binding to the *Glut4* promoter and Histone H3 acetylation in the *Glut4* promoter, that occur after exercise.
- (c) Activation of CaMK IV in C2C12 myotubes will increase the binding of MEF2A to the *Glut4* promoter and increase *Glut4* mRNA and protein levels.

To investigate these hypotheses, Wistar rats weighing ~200 g, were injected intraperitoneally with KN93 or vehicle and were exercised by swimming intermittently for 85 minutes carrying a load of 4% body weight. Rats that received vehicle but did not swim were used as controls. Triceps muscles were dissected from anaesthetised rats between 0 and 18 hours post-

exercise and chromatin immunoprecipitation assays were used to assess MEF2A binding and histone acetylation at the MEF2 binding site in the *Glut4* promoter. qRT-PCR was used to measure GLUT4 mRNA content and western blots were used to measure protein levels in these muscles. To investigate whether CaMK activation in skeletal muscle was sufficient to increase GLUT4 expression, constitutively active (CA) or dominant negative (DN) human CaMK IV proteins were expressed in C2C12 myotubes using recombinant adenoviruses.

The results show that CaMK II activity and *in vivo* acetylation of histone H3 at the *Glut4* promoter were increased ~1.8-fold ($P < 0.05$) immediately after exercise compared to controls. At 6 hours post-exercise, *Glut4* promoter bound MEF2A was ~4-fold ($P = 0.003$) and GLUT4 mRNA was ~2-fold ($P = 0.01$), higher than controls, while GLUT4 protein was elevated ~1.8-fold, 18 hours after exercise ($P = 0.005$). KN93 inhibited the increase in autonomous CaMK II activity and phosphorylation after exercise and significantly reduced the exercise-induced increases in DNA-bound MEF2A ($P = 0.007$), histone acetylation ($P = 0.01$) and GLUT4 protein ($P = 0.03$). C2C12 myotubes that expressed CA CaMK IV had ~ 2-fold more *Glut4*-promoter bound MEF2A ($P = 0.01$), GLUT4 mRNA ($P = 0.005$) and GLUT4 protein ($P = 0.003$) than DN CaMK IV expressing cells.

These results indicate that CaMK activity is sufficient for GLUT4 expression in C2C12 myotubes and is necessary for GLUT4 expression in rats in response to exercise. The data also suggest that CaMK may regulate GLUT4 expression by enhancing MEF2A access to its binding domain in the *Glut4* promoter by hyper-acetylating histones at the MEF2 binding site on the *Glut4* gene. Because increased expression of GLUT4 in skeletal muscle is associated with improved insulin sensitivity (108), the results of this investigation suggest that pharmaceuticals which activate CaMK II, increase in the binding of MEF2A to the *Glut4* promoter or increase acetylation of histones in the *Glut4* promoter, may have therapeutic potential for patients with type II diabetes.

CHAPTER ONE

Literature Review

1.1 Introduction

Type II diabetes mellitus is a non-communicable metabolic disease that is characterised by insulin resistance and varying degrees of insulin deficiency (237). Because patients with type II diabetes are resistant to insulin, they cannot adequately dispose of glucose from the circulation into tissues, which results in an acute energy deficit and a chronic state of hyperglycemia. Chronic hyperglycemia can damage blood vessels and cause blindness, renal failure, coronary artery disease or nerve disease (167). Recent reports estimated that diabetes accounted for 5.2% of all global deaths and 4.3% of all South African deaths during the year 2000 (14; 143; 172). The World Health Organisation (WHO) estimated that the global prevalence of Type II diabetes would double from 171 million in 2000 to 366 million in 2030 (214) and it is estimated that global costs associated directly with diabetes are currently 232 billion US dollars a year and may reach 302.5 billion a year by 2025 (47). It is now well known that the increasing incidence of diabetes worldwide is partly linked to a decline in physical activity. Indeed, 20% of all type II diabetes cases in South Africa were attributable to a lack of physical activity in the year 2000 (88). According to the World Health Survey conducted by the WHO in 2006, 43% of men and 48% of women in South Africa were reported to be physically inactive in 2003, compared to a global average of 17% and an African average of 10% (88). Clearly, diabetes is a growing socio-economic concern in South Africa and measures to combat its increase are needed urgently.

There is now vast evidence that regular exercise reduces the risk of developing type II diabetes and improves insulin sensitivity and glucose homeostasis in patients with the disease (103). There is mounting evidence that these benefits from exercise are attributed to elevated levels of the skeletal muscle glucose transporter protein (GLUT4), which is responsible for insulin-stimulated glucose uptake from the circulation (84). It has been shown that by raising GLUT4 levels in skeletal muscle, which is the main site for glucose uptake in the body, insulin-stimulated glucose disposal from the circulation is improved (24; 25; 108). The general purpose of this thesis was to investigate some of the molecular mechanisms by which exercise increases GLUT4 expression in skeletal muscle with the expectation that elucidating these mechanisms

would further our understanding of how exercise protects against developing type II diabetes and could potentially reveal novel targets for pharmaceuticals to treat the disease.

There is some indirect evidence that GLUT4 expression in skeletal muscle is mediated by Calcium/Calmodulin dependent protein kinase (CaMK) in response to elevated cytosolic calcium (Ca^{2+}) levels (150). However, it is not known whether CaMK also regulates the increase in GLUT4 protein content in response to exercise. The aim of this thesis therefore, was to investigate the role that CaMK plays in the exercise-induced increase in GLUT4 expression in skeletal muscle. To provide the necessary background information for the work presented in this thesis, a review of related literature is presented in this chapter. This review discusses the following topics:

- The mechanisms by which GLUT4 increases glucose transport in skeletal muscle
- The evidence that elevated muscle GLUT4 content improves insulin resistance
- The effects of exercise on GLUT4 expression in skeletal muscle
- The major transcription factors that regulate the *Glut4* promoters in response to exercise
- The potential role that chromatin remodelling plays in GLUT4 expression due to exercise
- The potential role that CaMK plays in GLUT4 expression in response to exercise
- The regulation of CaMK activity in skeletal muscle during exercise
- Other signals that may also regulate GLUT4 expression in response to exercise.

1.2 The mechanisms by which GLUT4 increases glucose transport in skeletal muscle

Skeletal muscle is important for glucose homeostasis because it is the major site for whole body glucose disposal from the circulation. Glucose transport into skeletal muscle occurs via facilitated diffusion by at least two of the major glucose transporter proteins namely, GLUT1 and GLUT4 (95). GLUT1 is found exclusively at the cell surface and is responsible for basal levels of glucose transport, while GLUT4 migrates between the cell interior and cell surface and participates in both basal glucose transport and insulin- or contraction-stimulated transport (118). As illustrated in Figure 1.1, during basal conditions, GLUT4 is located predominantly in an inactive state within intracellular membrane compartments (26). After a meal, insulin is released into the circulation and binds to a sarcolammal-bound insulin receptor (IR), triggering the propagation of a signalling cascade that ultimately causes that translocation of GLUT4 to a functional site in the sarcolemma. This signalling cascade involves insulin receptor substrate (IRS) 1/2, phosphatidylinositol 3-kinase (PI3 kinase), protein kinase B (PKB) and phosphoinositol

dependent kinase (PDK) 1/2 (4; 8; 170; 207). Individuals who are obese and/or have a sedentary lifestyle often develop disruptions in this signalling cascade, which results in the pathological condition known as insulin resistance and may lead to type II diabetes (176; 236).

INSULIN SIGNALLING CASCADE

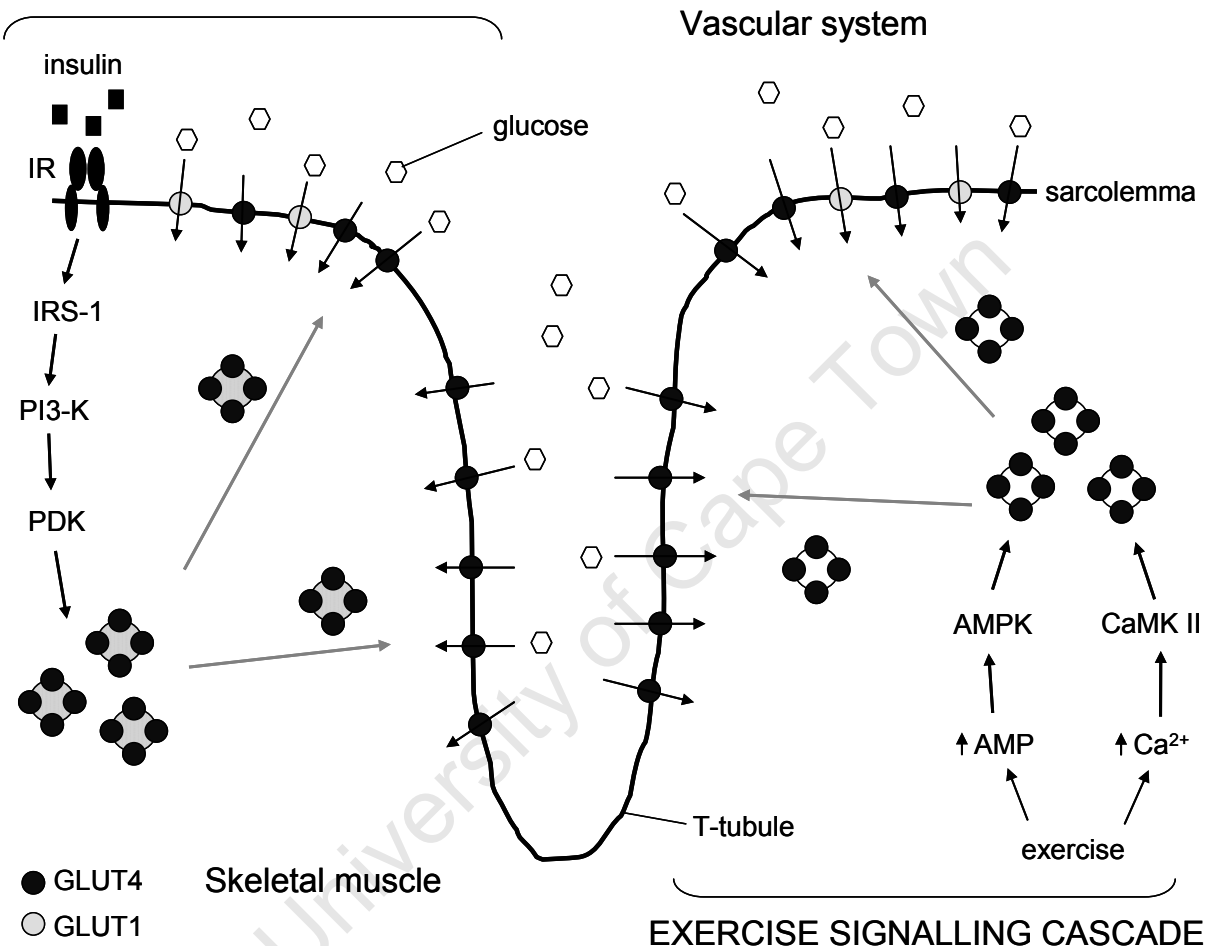


Figure 1.1. Glucose transport into skeletal muscle. Basal glucose transport from the circulation into skeletal muscle occurs via GLUT1 and GLUT4 that reside at the sarcolemma. Enhanced glucose transport occurs when insulin (left side) or muscle contraction (right side) cause additional GLUT4 proteins to translocate from intra-cellular vesicles to the sarcolemma. Exercise and insulin function via different mechanisms and target different intracellular pools of GLUT4. The insulin signalling cascade involves a membrane bound insulin receptor (IR), insulin receptor substrate (IRS)-1, phosphatidylinositol 3-kinase (PI3-K) and phosphoinositide dependent kinase (PDK). Contraction stimulated GLUT4 translocation involves calcium (Ca^{2+})/calmodulin dependent protein kinase (CaMK) II and adenosine monophosphate (AMP) dependent protein kinase (AMPK). Diagram modified from Hayashi *et al.* (66).

Muscle contraction during exercise also stimulates GLUT4 to translocate to the sarcolemma and increases glucose transport into skeletal muscle (66; 164; 197). Glucose uptake in response to exercise involves AMP-dependent protein kinase (AMPK) and CaMK II (224), is independent of IRS-1/2 or PI3 kinase (52; 78; 177) and utilizes a different pool of intracellular GLUT4 (26). These observations suggest that the mechanism by which exercise increases glucose uptake is different to that of insulin (66; 140; 230). Indeed a number of studies have shown that individuals with defective insulin-stimulated glucose uptake, have normal contraction-stimulated glucose uptake and the decline in glucose transport capacity due to defects in insulin signalling may be compensated for by exercise (15; 66). Results from such studies provide the scientific basis for prescribing exercise to manage the hyperglycemia that is usually associated with insulin resistance and type II diabetes (103).

1.3 The evidence that elevated muscle GLUT4 content improves insulin resistance

In many human cases and animal models of type II diabetes (e.g. streptozotocin induced diabetic rats, high fat-fed rats and obese diabetic rats), skeletal muscle GLUT4 content is reduced, suggesting that the decline in insulin sensitivity may be attributed to decreased expression of GLUT4 (16; 36; 42; 90; 117; 185). In other human cases of type II diabetes and in some animal models of insulin resistance (e.g. obese Zucker rats and db/db mice), total GLUT4 levels in skeletal muscle are not compromised, suggesting that insulin resistance may also be caused by defects in the mechanism responsible for translocating GLUT4 to the cell surface (30; 43; 62; 92; 99; 158). Whether GLUT4 levels are compromised in type II diabetes or not, there is now mounting evidence to suggest that insulin resistance can be ameliorated by increasing GLUT4 protein content in skeletal muscle. For example, over-expression of GLUT4 in streptozotocin-induced diabetic rats, db/db diabetic mice or high fat-fed rats results in improved insulin sensitivity and enhanced whole body glucose disposal (51; 81; 108). Of particular note is the finding that over expression of GLUT4 specifically in skeletal muscle resulted in improved insulin sensitivity and enhanced whole body glucose disposal in transgenic streptozotocin-induced diabetic mice (108). This finding suggests that interventions which enhance GLUT4 expression in skeletal muscle may serve as an effective therapy to treat insulin resistance.

1.4 The effects of exercise on GLUT4 expression in skeletal muscle

It is now well established that regular exercise is an effective way to increase GLUT4 protein content in both human and rat skeletal muscle. Furthermore, exercise improves whole body insulin sensitivity and glucose homeostasis in patients with type II diabetes (80; 159; 164). Transcription of the *Glut4* gene increases ~1.8 fold and GLUT4 mRNA levels rise 2-2.5-fold within 3 hours after a single bout of exercise in rat and human skeletal muscle (49; 101; 141; 164). Following the increase in transcript levels, GLUT4 protein content increases by 1.5-2-fold, within 16-24 hours after a bout of exercise (101; 164). The increase in GLUT4 protein in rat and human skeletal muscle accumulates with consecutive daily bouts of exercise but soon reached a steady state level that remains ~2-3 fold higher than sedentary controls, as long as training persists (43; 49; 61; 74; 75; 77; 164; 169; 193; 231).

The elevated GLUT4 content observed after an acute bout of exercise or after short term training (5 days to 5 weeks) is transient and returns to control levels within 42-48 hours after the last bout of exercise (49; 73; 165). In individuals who have a history of participating in endurance training, the elevated levels of GLUT4 appear to persist for longer after the cessation of exercise: In one study involving endurance and strength trained individuals, GLUT4 content was reported to be unaltered after 14 days of inactivity (76). In two other studies on endurance trained athletes, only a 17% and a 33% decrease in GLUT4 contents were reported within 6 and 10 days of inactivity respectively (120; 205). Therefore, long term endurance training appears to be an effective way to maintain elevated GLUT4 protein levels in skeletal muscle.

Traditionally, low intensity exercise at 50-70% of peak oxygen consumption (VO_2 peak) lasting more than 30 minutes was thought to be the most effective way to increase GLUT4 protein in skeletal muscle and was therefore the recommended form of exercise to treat type II diabetes (33; 61; 80; 193). Consistent with this belief, the model used most extensively to study GLUT4 expression in rats consisted of swimming for 2 bouts of 3 hours (49; 164; 193). However, some more recent studies have shown that higher-intensity intermittent exercise performed over a shorter duration is equally as effective at increasing GLUT4 protein content. For example, rats which performed 5 x 17 minute bouts of swimming carrying a load of 5% body weight, had similar elevations in GLUT4 expression compared to rats which performed lower-intensity prolonged swimming lasting 6 hours (193). Similar results have been shown in humans where 60 minutes of cycling at 40% VO_2 peak caused an equivalent increase in GLUT4 protein compared to 27 minutes at 80% VO_2 peak (101). Therefore it is now accepted that both prolonged low-

intensity and short-duration, high-intensity intermittent exercise induce rapid but transient increases in GLUT4 protein that accumulate with repetitive daily bouts of exercise and is regulated at the transcriptional level.

In insulin resistant or diabetic individuals, exercise training increases insulin-stimulated glucose disposal and GLUT4 protein levels but has no effect on insulin stimulated PI3 kinase activity (25). These findings are consistent with those using insulin resistant obese Zucker rats (24) and suggest that, in insulin resistant individuals, exercise increases insulin sensitivity primarily by increasing GLUT4 protein content. Interestingly, in animals or individuals that do not have insulin resistance, the increase in insulin sensitivity with exercise training appears to be mediated by an increase in the abundance and activity of both GLUT4 and the intermediates involved in insulin signalling (20; 91; 93; 114). These studies illustrate the importance of physical activity for preventing and treating diabetes. However, there are numerous circumstances where individuals become diabetic or insulin resistant because they are unable to exercise. In these cases, pharmaceuticals which increase GLUT4 expression by activating the same pathways as exercise may be of immense therapeutic value. For these drugs to be developed, it is first essential to characterise the molecular mechanisms and signalling intermediaries that regulate GLUT4 expression in response to exercise.

1.5 The major transcription factors that regulate GLUT4 expression in response to exercise

Research aimed at understanding how exercise increases GLUT4 expression began by identifying domains in the human *Glut4* promoters that are responsive to exercise. Studies in transgenic mice carrying various constructs of the human *Glut4* promoter fused to a CAT reporter gene revealed that the region within 895 bp of the transcription start site is sufficient to increase skeletal muscle CAT expression in response to exercise (116). Furthermore this region of the *Glut4* promoter is highly conserved in humans, rats and mice (82). Two exercise-responsive domains that are illustrated by the shaded regions in Figure 1.2 were identified within this region: A myocyte enhancer factor (MEF) 2 binding site between bases -482 and -490 that is conserved in rats (bases -457 to -466) and mice (bases -429 to -437) (82; 110; 151; 194; 202) and a novel binding domain called Domain I, which lies upstream of the MEF2 site, between bases -712 and -742 (154). Mutation of either the MEF2 site in the rat or human *Glut4* promoters or Domain I (between bases -725 and -729) in the human *Glut4* promoter prevented transcription of a reporter

gene, indicating that these binding sites are necessary for normal GLUT4 expression in skeletal muscle (110; 151; 154; 194).

Electrophoretic mobility shift assays (EMSA) have shown that a novel protein called GLUT4 enhancer factor (GEF), binds Domain 1 in the human *Glut4* promoter (96; 154) and that this binding increases after a bout of exercise in humans (122). EMSA using isoform-specific antibodies has also revealed that two isoforms of MEF2, namely MEF2A and MEF2D, bind to the MEF2 site in the human and rat *Glut4* promoters as a MEF2D homodimer or a MEF2A/MEF2D heterodimer (110; 122; 122; 133; 194) and that the binding of MEF2A/MEF2D increases after a bout of exercise (122) or when muscles are made to contract by electrical stimulation (184). Furthermore, over-expression of either MEF2A, MEF2D or GEF in COS 7 cells (a monkey kidney cell line that is used as a general cell culture model), results in increased activity of the human *Glut4* promoter (96). Studies in COS 7 cells show that both MEF2A and MEF2D physically interact with GEF (96) and that MEF2A binding to its binding site increases the affinity of GEF for domain 1 (186), suggesting that MEF2 proteins and GEF co-operate to regulate GLUT4 expression. Support for this theory came from the observation that simultaneous over-expression of MEF2A and GEF in COS 7 cells increased human *Glut4* promoter activity considerably more than the sum of activities when the two factors were expressed separately (96). Interestingly, simultaneous expression of MEF2D and GEF did not show a co-operative effect on *Glut4* promoter activity. Based on these findings, the complex formed by the MEF2A/MEF2D heterodimer and GEF at the *Glut4* promoter appears to play an important role in increasing *Glut4* gene expression after exercise (122; 133; 154). Although there is limited information on how MEF2A and GEF interact when bound to the *Glut4* promoter, one possible mechanism that would facilitate the formation of a complex could involve DNA looping, which would bring the two respective binding sites into close proximity.

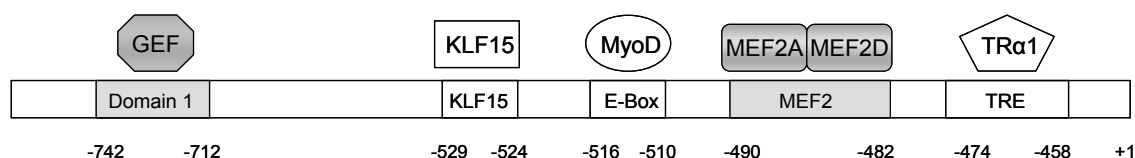


Figure 1.2. Regulatory sequences in the human *Glut4* promoter and the factors that bind to them in skeletal muscle. Exercise increases the binding of GLUT4 enhancer factor (GEF) and a myocyte enhancer factor (MEF) 2 A/D heterodimer to the indicated binding domains in the *Glut4* promoter. Kruppel like factor (KLF)-15, MyoD and thyroid hormone receptor (TR) α 1 also bind the *Glut4* promoter, however the role of these factors during exercise is uncertain. Numbers indicate the nucleotide position from the transcription start site (+1). TRE = thyroid hormone receptor element.

There are also other conserved binding domains in the human, rat and mouse *Glut4* promoters that are in close proximity to the MEF2 domain: an E-box which binds the muscle regulatory factor MyoD and a thyroid hormone receptor element (TRE) that binds thyroid hormone receptor (TR α 1) (see Figure 1.2) (179; 201). These binding sites in conjunction with the MEF2 element, constitute an enhancer sequence that is markedly activated during muscle regeneration and perinatal life in rats (17; 134; 178). Although co-expression of MyoD, TR α 1 and MEF2A in L6E9 muscle cells can potentially augment the rat *Glut4* promoter (179), they are unlikely to contribute towards GLUT4 expression in response to exercise in adult skeletal muscle. In adult muscle, there is low expression of the muscle regulatory factors that regulate the E-box (38; 98) and deletion of the TRE site results in a 2-fold increase in rat *Glut4* promoter activity, suggesting that this site may in fact play a negative regulatory role in adult muscle, presumably by factors other than TR α 1 (134).

Kruppel like factor 15 (KLF15) is another transcription factor that binds to a region near the MEF2 domain in the rat *Glut4* promoter (-499 to -503) that is conserved in humans and mice (54; 238). KLF-15 is highly expressed in skeletal muscle and acts synergistically with MEF2 to potentially increase GLUT4 expression in C2C12 myotubes (54). However, it is unclear whether KLF-15 is activated by exercise or whether it is involved in exercised-induced GLUT4 expression in human skeletal muscle.

In summary, there is much evidence which suggests that the MEF2A/MEF2D heterodimer and GEF bind to and regulate the human, rat and mouse *Glut4* promoters in response to a bout of exercise. However, the mechanisms that are responsible for these actions have not been well characterised. In response to this lack of knowledge, this thesis investigated the role that CaMK activation and chromatin remodelling might play on the *in vivo* binding of MEF2A to the *Glut4* promoter in skeletal muscle.

1.6 The potential role that chromatin remodelling plays in GLUT4 expression in response to exercise

Access of transcription factors and RNA polymerases to their binding domains on DNA is often regulated by the structure of the chromatin surrounding a gene. At a primary structural level, DNA is packaged into nucleosomes to form chromatin. Nucleosomes consist of 146 bp of DNA that wraps around a histone octamer composed of two molecules each of histones H2A, H2B, H3

and H4 that are separated from neighbouring nucleosomes by a region of linker DNA as shown in Figure 1.3 (6). Nucleosomes occupy defined regions of DNA within the promoters and enhancers of many genes and often restrict access of transcription factors to their binding domains (196; 218). The degree of compactness of the chromatin is regulated by post-translational modifications of the histones e.g. by acetylation, methylation or phosphorylation, which alters the electrostatic interaction between the DNA and histones (9; 102; 196; 218). For example, hyper-acetylation of the lysine residues on the N-terminal tails of histone H3 or H4 neutralises the positive charge on these lysine residues. As illustrated in Figure 1.3, this weakens the electrostatic interaction between the histones and the negatively charged phosphate backbone of the DNA, thus loosening chromatin compactness and facilitating access of transcription factors and RNA polymerases to their binding domains (56; 58; 144; 146).

Histones are acetylated by factors that possess intrinsic histone acetyl transferase (HAT) activity such as p300, CREB-binding protein (CBP) and p300/CBP-associated factor (PCAF) (162). With regard to genes that are regulated by MEF2, it has been reported that binding of the transcriptional co-activator, peroxisome proliferator-activated receptor gamma coactivator -1 α (PGC-1 α), to transcription factors such as MEF2, creates a conformational change in PGC-1 α that facilitates the docking of CBP/p300, which then acetylates histones near MEF2 binding domains in gene promoters (Figure 1.3) (97; 130; 147; 161). CBP/p300 also associates directly with MEF2A and MEF2C, which markedly potentiates MEF2 transcriptional activity (32; 180). In addition to acetylating histones within gene promoters, p300 also acetylates MEF2C at several sites within its transactivation domain, which increases its DNA binding efficiency (3; 115). The lysine residues of MEF2C that are acetylated by p300 are conserved in MEF2A and MEF2D from various species, which suggests that acetylation by p300 may be a general mechanism to increase MEF2-DNA binding (115).

There are also reports that hyper-acetylated histones themselves may recruit transcriptional co-activator complexes to gene promoters because HATs possess bromodomains that interact specifically with acetylated lysines in histone H3 and H4 tails (34; 100; 189). Therefore the initial recruitment of HATs to the MEF2 site in response to a stimulus likely involves the MEF2 transcription factor itself; however, once hyper-acetylation occurs, transcription machinery may be assembled more rapidly because the hyper-acetylated histones also recruit or stabilise transcription factors on chromatin to maintain a high transcription rate.

In contrast to the increased rates of transcription that are associated with acetylated histones, deacetylation of the lysine residues restores the positive charge on the histone tails, causing them to interact more tightly with the DNA backbone, thus restricting access of transcription factors to their binding domains and preventing transcription (6; 195). Class II histone deacetylases, such as HDAC4 or HDAC5, bind to MEF2 factors and repress transcription of MEF2 regulated genes by deacetylating histones that surround MEF2 binding domains (Figure 1.3) (113). Zhao *et al.* (235) demonstrated that HDAC4 could also stimulate the attachment of small ubiquitin-related modifiers (SUMOs) to Lys 424 of MEF2D in 293 cells, which was associated with decreased MEF2 activity in 10T1/2 fibroblasts. Several studies report that lysine SUMOylation negatively regulates the activity of transcription factors (204). Interestingly lysine 424 on MEF2D is also acetylated by p300/CBP; an event which is associated with enhanced transcriptional activity (115; 235), indicating a possible interplay between HDAC4 and p300/CBP at Lys 425 of MEF2D. Thus, HDACs repress MEF2 activity both by SUMOylation of MEF2 and by deacetylating histones within promoters of MEF2 regulated genes, while HATs de-repress MEF2 by acetylating these histone residues and increase MEF2 binding ability by acetylating MEF2 directly.

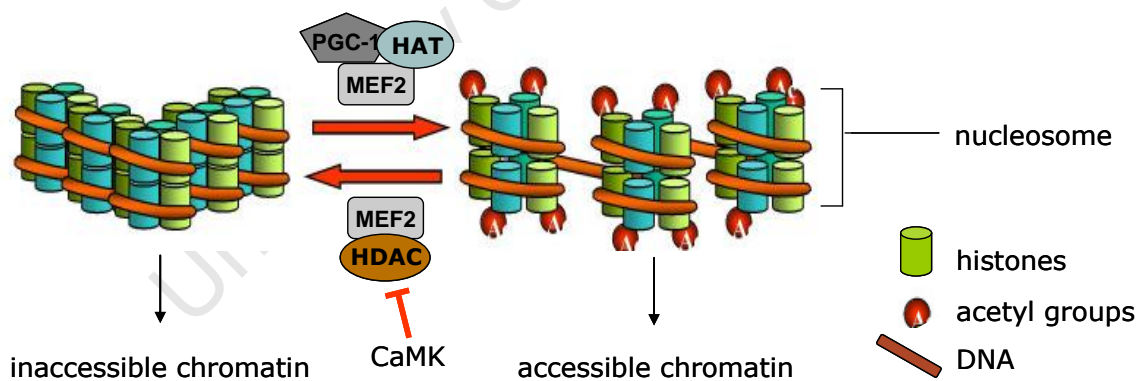


Figure 1.3. Chromatin is packaged into nucleosomes. Nucleosomes consist of DNA wrapped around a histone octamer. Histone deacetylases (HDACs) bind to myocyte enhancer factor (MEF) 2 and deacetylate histones near MEF2 binding domains in gene promoters, creating compact chromatin, which restricts access for transcription factors to their binding domains. Ca^{2+} /Calmodulin dependent protein kinase (CaMK) II (see section 1.7) dissociates HDACs from MEF2 which facilitates peroxisome proliferator-activated receptor gamma coactivator -1 α (PGC-1 α) to bind to MEF2 and recruit factors having histone acetyl transferase (HAT) activity. HATs acetylate histones, which weaken the interactions between histones and DNA, creating chromatin that is more accessible to transcription factors.

There are several lines of evidence which lend support to the hypothesis that, during exercise, the *Glut4* promoter is regulated by factors that can remodel chromatin at the MEF2 binding site. For example, expression of an HDAC5 that constitutively localises in the nucleus results in a 3-fold decrease in GLUT4 protein in cardiac muscles from transgenic mice (31). Furthermore, a recent study by Sparling *et al.* (186) showed that HDAC5 associates with both MEF2A and GEF *in vitro* in COS-7 cells and co-immunoprecipitates *in vivo* with chromatin containing the MEF2 and GEF binding sites in the *Glut4* promoter in adipocytes. These authors also showed that over-expression of HDAC5 in COS-7 cells, reduces the increase in *Glut4* promoter activity in response to over-expression of MEF2A and GEF factors. During exercise HDACs dissociate from MEF2 and are shuttled from the nucleus to the cytoplasm, which compromises their ability to repress nuclear transcription factors (122). Collectively, these recent findings suggest that HDAC5 forms a complex with MEF2 and GEF at the *Glut4* promoter which represses GLUT4 expression and exercise relieves this repression by exporting the deacetylase from the nucleus.

The pattern of acetylation of histones in the *Glut4* promoter has not been characterised to date and the effect of exercise on this acetylation is not known. Nor is it known whether GLUT4 expression is regulated by HATs. However, there is some evidence suggesting that GLUT4 expression after exercise may be regulated by PGC-1 α . A single bout of exercise causes a rapid increase in PGC-1 α protein content that is evident within 3 hours after exercise (5; 223). Exercise also causes nuclear PGC-1 α to associate with MEF2 factors (121). Furthermore, over expression of PGC-1 α in L6 myotubes causes a marked up-regulation of *Glut4* promoter activity (130). Experiments to confirm whether PGC-1 α plays a role in exercise-induced GLUT4 expression are therefore warranted. Additionally, the influence of PGC-1 α and HATs such as p300 on the acetylation state of histones in the *Glut4* promoter should be investigated.

1.7 The potential role that CaMK plays in GLUT4 expression in response to exercise

Signals that regulate whether MEF2 associates with HATs or HDACs are likely to control MEF2 binding to the *Glut4* promoter and may therefore play an important role in regulating GLUT4 expression. There are reports that CaMK II, which is activated by the rise in cytosolic Ca²⁺ during muscle contraction (see section 1.8, (174)), increases MEF2 transcriptional activity in skeletal muscle (155) by phosphorylating HDACs within HDAC/MEF2 complexes (124). The phosphorylated deacetylase then dissociates from MEF2 and is exported from the nucleus by the

chaperone protein 14-3-3 (57; 124; 126; 206). It can be speculated that the liberated MEF2 would then be free to form complexes with co-activator molecules such as PGC-1 α and/or p300, which would facilitate MEF2 acetylation and its binding to the *Glut4* gene. Furthermore this complex could facilitate the acetylation of histones within the *Glut4* gene resulting in increased accessibility of transcription factors and RNA polymerases to the *Glut4* promoter.

The speculation that CaMK activation will lead to remodelling of chromatin is consistent with results from an experiment in L6 myotubes showing that CaMK is necessary for the acetylation of histones surrounding MEF2 binding sites within the promoters of myogenin and muscle creatine kinase (MCK) genes in response to Arg-Vasopressin (AVP) treatment (181). In these experiments AVP also increased the binding of MEF2 to its binding domain in these promoters and increased MCK and myogenin protein levels in a manner that was dependent on CaMK activity. Ojuka *et al.* (150) also provided evidence that CaMK was involved in GLUT4 expression: They incubated L6 myotubes with caffeine to mimic the rise in cytosolic Ca²⁺ that normally occurs during muscle contraction and noticed that GLUT4 protein levels increased after 5 days of incubation. When either KN93 (which inhibits CaMK II activity) or dantrolene (which inhibits Ca²⁺ release from the sarcoplasmic reticulum) were added to the medium, the increase in GLUT4 was abolished, suggesting that Ca²⁺ might mediate GLUT4 expression in skeletal muscle via activation of CaMK. Rose *et al.* (174) recently showed that CaMK II was activated in human skeletal muscle during a bout of sub-maximal exercise, however it is not known whether this increase influences the binding of MEF2 to its site in the *Glut4* promoter or the acetylation of histones within this site on the gene. This thesis aims to address the role that CaMK plays in GLUT4 expression during exercise with particular reference to its potential influence on the binding of MEF2A to its site in the *Glut4* promoter and the acetylation of histones near this site.

1.8 Activation of CaMK II in skeletal muscle during exercise

To design experiments to test whether CaMK is involved in exercise-induced GLUT4 expression, a clear understanding of the regulation of CaMK activity in skeletal muscle is required. CaMK II, which is the predominant CaMK isoform found in skeletal muscle (173; 174), is activated by elevations in cytosolic Ca²⁺ (72). During muscle contraction, each wave of depolarisation of the sarcolemma causes an increase in cytosolic Ca²⁺, which is rapidly buffered by membrane pumps and Ca²⁺ binding proteins, resulting in a series of cytosolic Ca²⁺ spikes (111; 129). The information carried by the frequency of these Ca²⁺ transients is transduced by

calmodulin (CaM), which binds Ca^{2+} and undergoes a conformational change that enables it to bind to and activate CaMKs (21). CaMKs in turn translate the Ca^{2+} message by phosphorylating target substrates on Serine or Threonine residues (72).

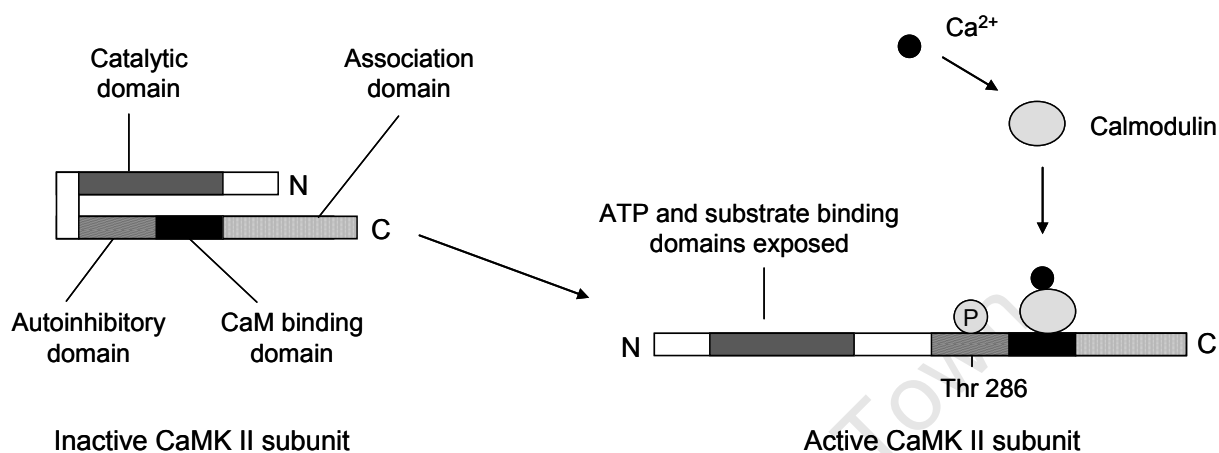


Figure 1.4. Activation of CaMK II subunits. During basal conditions CaMK II subunits are repressed by an autoinhibitory domain that acts as a pseudosubstrate and blocks the ATP- and substrate- binding sites in the catalytic domain (left side). Binding of calmodulin that has been activated by calcium (Ca^{2+}) causes a conformational change in CaMK II that exposes the ATP- and substrate- binding domains resulting in maximal activity of the subunit and enables Thr 286 to be phosphorylated by another activated subunit in the holoenzyme (right side).

The functional properties of CaMK II are related to its multimeric structure and numerous reviews document in detail the structure and mechanisms of activation of CaMK II (22; 72; 79). CaMK II exists as an 8-12 subunit hetero- or homo-multimeric holoenzyme (183; 198). As illustrated in Figure 1.4, each subunit has a catalytic domain that contains ATP- and substrate-binding sites; an autoinhibitory domain that contains a CaM binding site and an association domain that is necessary for the formation of the holoenzyme (72; 157; 183). The autoinhibitory region acts as a pseudo-substrate that binds to the substrate binding pockets in the catalytic domain to maintain CaMK in an auto-inhibited state as seen in Figure 1.4 (29; 89). Adjacent to the inhibitory region is a CaM binding domain. Binding of Ca^{2+} /CaM to this domain causes a conformational change in the enzyme that exposes the ATP and substrate binding sites and activates the subunit. This conformational change also exposes Threonine (Thr) 286 in the inhibitory domain (28; 157). As illustrated in Figure 1.5, when two adjacent subunits in the CaMK II holoenzyme are bound by CaM, one subunit will phosphorylate the neighbouring subunit on its exposed Thr 286 residue. Phosphorylation at Thr 286 increases the enzyme's

affinity for CaM: a state known as ‘CaM trapping’. Phosphorylation also prevents the interaction of the inhibitory loop with the catalytic domain, causing the ATP and substrate binding sites to remain exposed after $\text{Ca}^{2+}/\text{CaM}$ dissociates (29; 64). This results in $\text{Ca}^{2+}/\text{CaM}$ independent (autonomous) activity that is retained until the subunits are dephosphorylated by a phosphatase (29; 64; 83; 104). Because exercise increases cytosolic Ca^{2+} levels, activates CaM and phosphorylates CaMK II at Thr 286, the activation of CaMK II by exercise is likely to occur by the mechanism described above (174). Indeed, Rose *et al.* (174) recently showed that autonomous CaMK II activity in humans increases ~9 fold at the onset of a bout of sub maximal exercise, dropped to ~2-3 fold higher than basal after 10 minutes and remained elevated at that level for the duration of the 60 minute exercise.

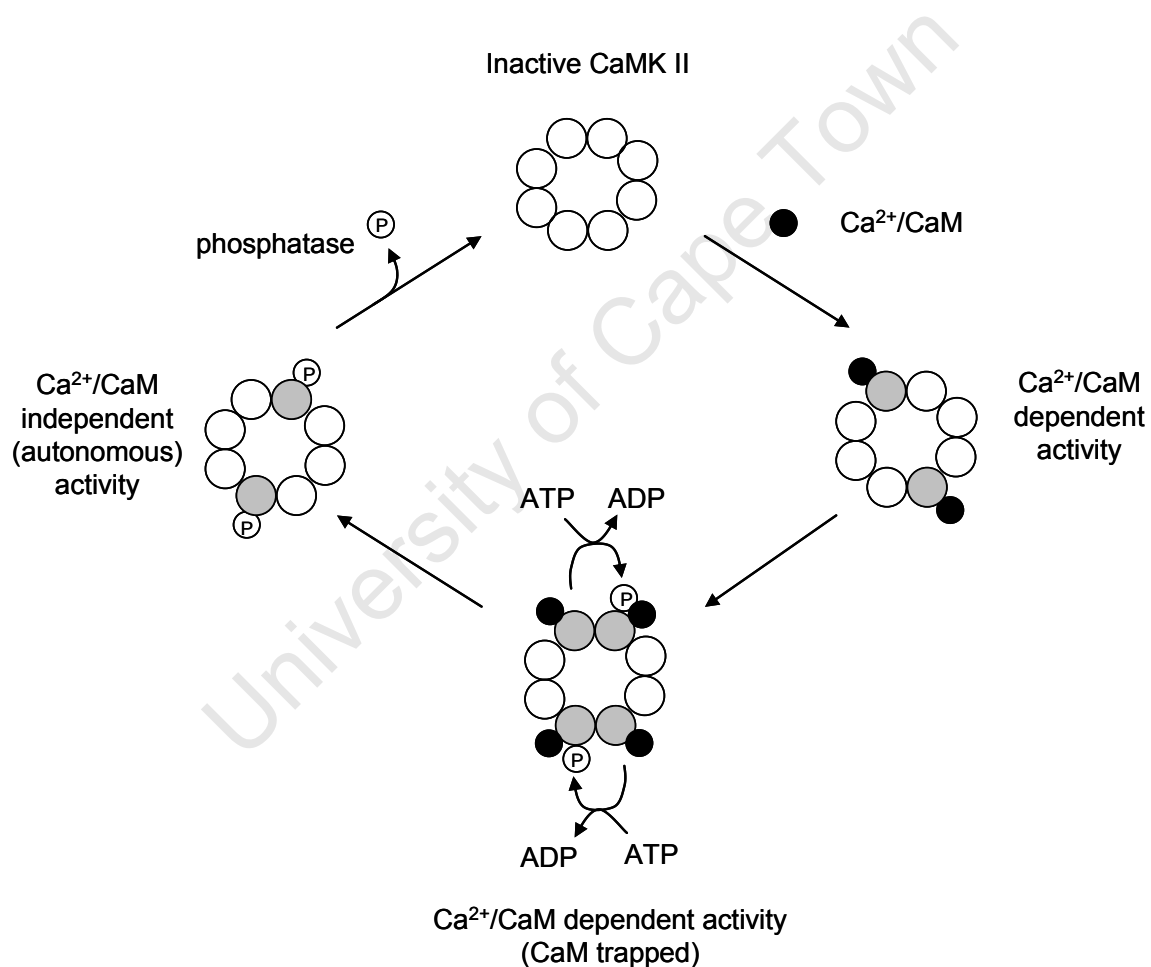


Figure 1.5. CaMK II undergoes $\text{Ca}^{2+}/\text{CaM}$ dependent and independent (autonomous) activity. The CaMK II holoenzyme is depicted as an octamer with shaded circles representing active subunits. $\text{Ca}^{2+}/\text{CaM}$ binds to a subunit and activates it. $\text{Ca}^{2+}/\text{CaM}$ -bound subunits then autophosphorylate neighbouring $\text{Ca}^{2+}/\text{CaM}$ -bound subunits within the holoenzyme. Phosphorylation results in enhanced $\text{Ca}^{2+}/\text{CaM}$ affinity (CaM trapped) and confers a state of autonomous activity after $\text{Ca}^{2+}/\text{CaM}$ dissociates, which persists until the subunits are dephosphorylated by a phosphatase. Diagram adapted from Hudmon and Schulman (79).

Much of what is known about CaMK II function has been deduced using compounds which pharmacologically inhibit CaMK II activity such as the KN range of inhibitors. KN62, and its more water soluble counterpart, KN93, are potent and specific inhibitors of CaMK II that have little or no influence on other protein kinases such as protein kinase A (PKA) or C (PKC) (190). Although the exact mechanism of inhibition is not clearly understood, these compounds appear to compete with $\text{Ca}^{2+}/\text{CaM}$ for its binding site on the CaMK II subunit. Once bound, they prevent new activation of the subunit but have little effect on the activity of subunits that have already been activated by phosphorylation (190; 199). KN93 has been widely used to inhibit CaMK II activity in cultured muscle cells, in isolated muscles and in live animals (10; 137; 150; 221). It has however been reported that, in addition to inhibiting CaMK II, KN93 also inhibits voltage-dependent K^+ currents (166) and L-type calcium channel activity (48). Therefore care must be taken to use appropriate controls when using this compound in experiments. For this purpose KN92, an analogue of KN93 which inhibits voltage-dependent K^+ currents and L-type calcium channel activity but does not inhibit CaMK activity, is widely used (48; 166). Using both of these inhibitors, Mu *et al.* (135) recently demonstrated that KN93 inhibited MEF2 transcriptional activity in muscle by inhibiting CaMK II activity and not through its other secondary effects.

CaMK IV is another multifunctional calmodulin kinases that belongs to the same family of serine-threonine kinases as CaMK II and has a very similar domain structure (72). Chatila *et al.* (18) have demonstrated that mutants of the CaMK IV isoform, which lack the autoinhibitory domain (truncated at Leucine (Leu) 317), are active in skeletal muscle even in the absence of Ca^{2+} . Conversely, a mutation of Threonine to Alanine at amino acid 200 in the kinase domain completely prevented CaMK IV activity even in the presence of $\text{Ca}^{2+}/\text{CaM}$ or when the kinase was truncated at Leu 317. These constitutively active (CA) and dominant negative (DN) mutations of CaMK IV have been widely used in skeletal muscle to study CaMK function (59; 63; 225). Wu *et al.* (225) for example, showed that transgenic mice expressing a CA CaMK IV had augmented mitochondrial biogenesis and increased activities of mitochondrial enzymes. Furthermore, expression of a CA CaMK IV in C2C12 myotubes was equally as effective as other CaMK isoforms in activating transcription of a myoglobin promoter (225). Over expression of a CA CaMK IV in mice, rat muscle or C2C12 myotubes increased the abundance of PGC-1 α and the activity of the PGC-1 α promoter (60; 63; 225). This evidence shows that ectopically expressed CA CaMK IV that lacks the autoinhibitory domain is functional in skeletal muscle.

CaMK IV is found endogenously in neurons, developing T lymphocytes and post meiotic male germ cells (41; 128; 148) but not in skeletal muscle (1; 173; 174). This raises some concern as to whether ectopic expression of CaMK IV is a valid experimental design to study the role of CaMK in muscle. Numerous reasons have been advanced for the use of CaMK IV as a suitable surrogate for CaMK II in muscle. Firstly, the mechanisms of activation of both CaMK II and CaMK IV are similar. Both kinases are activated by the binding of $\text{Ca}^{2+}/\text{CaM}$ to the CaM binding domain, which displaces an autoinhibitory pseudo-substrate and exposes the ATP- and substrate-binding domains (72). Additionally, residues in both kinases are phosphorylated leading to a state of autonomous activity that is independent of $\text{Ca}^{2+}/\text{CaM}$. Whereas CaMK II undergoes autophosphorylation at Thr 286 by adjacent CaMK subunits in the holoenzyme, CaMK IV is phosphorylated by CaMKK at Thr 200 (18; 28; 29; 79; 157; 182; 200). Secondly, and more importantly, the substrate motifs that CaMK II and CaMK IV recognise are also similar¹ (211), suggesting that they may phosphorylate common proteins. Indeed, both CaMK II and CaMK IV phosphorylate HDAC4 on Ser 467 and Ser 632 (7; 234) and HDAC5 on Ser 259 and Ser 498 (109; 124). Furthermore phosphorylation of HDACs by both CaMK II and IV leads to a dissociation of the HDAC from MEF2, export of the HDAC from the nucleus and an activation of MEF2 transcriptional activity (7; 109; 113; 124; 234). Numerous other transcription factors are also phosphorylated by both CaMK II and CaMK IV, for example cAMP response element binding protein (CREB) on Ser 133 (119; 191), activating transcription factor-1 (ATF-1) on Ser 63 (192), CAAT- enhancer binding protein (c/EBP β) on Ser 276 (209; 232) and serum response factor SRF on Ser 103 (40; 131). The above reasons, coupled with difficulty of making CA and DN forms of the CaMK II holoenzyme and the ease at which CA and DN forms of CaMK IV can be generated, have popularised the use of CaMK IV as a surrogate for CaMK II in muscle.

1.9 Other signals in addition to CaMK II may also regulate GLUT4 expression during exercise

Exercise activates numerous other signals in skeletal muscle which may also influence *Glut4* gene expression. These include adenosine monophosphate (AMP)-activated protein kinase (AMPK), p38 mitogen activated protein kinases (MAPK) and Calcineurin (213; 216; 227).

¹ Substrate motifs for CaMK II = Hyd-X-ARG-X-X-Ser/Thr and CaMK IV = Hyd-X-Arg-NB-X-Ser/Thr-Hyd, where NB = non basic aa, Hyd = Hydrophobic aa and X = any aa.

AMPK is a signalling intermediate that is activated by the rise in AMP that occurs during moderate and high, but not low, intensity exercise (19; 163; 187; 217). Over-expression of a CA form of AMPK α (the AMPK subunit that contains the kinase domain) in skeletal muscle cells, increases GLUT4 expression, suggesting that AMPK activation may also signal to increase GLUT4 expression during exercise (44). Further evidence which suggests that AMPK α increases GLUT4 expression during exercise comes from experiments using 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), which mimics AMP and activates AMPK α 2, the AMPK α isoform that is activated in muscle by endurance exercise (138; 217). Injection of rats with AICAR or *in vitro* incubation of rat muscles with the compound results in increased expression of GLUT4 in skeletal muscle (69; 138; 150; 215). AMPK α 2 however, does not appear to be necessary for the increase in GLUT4 expression during exercise. In numerous reports in transgenic animals, ablation of AMPK α 2, attenuated the increase in GLUT4 by AICAR but had no effect on the increase in GLUT4 expression by exercise (70; 87; 168). These experiments that use AMPK α 2 knock out mice do not exclude the possibility that other signals or other isoforms of AMPK α might have played an unusually predominant role in GLUT4 expression to compensate for the lack of AMPK α 2 activity. Indeed exercise activates CaMK II or p38 which may also increase GLUT4 expression (174; 212). Furthermore, although the AMPK α 1 isoform is not activated in wild type animals during endurance exercise, it is activated in AMPK α 2 knockout mice by treadmill running (94). Taken together, these data provide convincing evidence that AMPK α 2, which is activated by endurance exercise, increases GLUT4 expression, however, there is also evidence that the AMPK α 2 isoform does not appear to be necessary for exercise to increase GLUT4 protein content.

In light of the findings that both AMPK and CaMK increase GLUT4 expression in muscle, Ojuka *et al.* (149; 150) proposed the following hypotheses: a) In conditions of brief low-intensity exercise training, when the concentrations of high-energy phosphates are stable or only marginally decreased, GLUT4 increases only modestly (45; 149). Under these conditions cytosolic Ca²⁺, acting via CaMK, may act as the primary stimulus for GLUT4 expression. b) When exercise is conducted at higher intensities or for prolonged periods, the ATP/AMP ratio in muscle is markedly reduced and AMPK activation and GLUT4 expression are significantly elevated (45; 171; 193). Under these conditions, the GLUT4 response to training may be due to both the Ca²⁺ and AMP stimulated pathways. These hypotheses however, have not been fully tested.

Recent evidence suggests that there may be cross-talk between CaMK II and AMPK activation pathways. This evidence stems from experiments using *ex vivo* soleus muscle preparations, in which incubation with sub-contraction levels of caffeine resulted in an increase in AMPK α 1 activity in a manner that was preventable by KN93 (an inhibitor of CaM binding) or STO-609 (an inhibitor of CaMKK activity) (85). Similarly AMPK α 1 and AMPK α 2 were activated in *ex vivo* soleus muscles by low intensity tetanic contractions in a manner that was also preventable by KN93 and STO-609 after 2 minutes of contractions (86). Interestingly AMPK phosphorylation was not prevented by KN93 after 10 minutes of contractions or during contractions at high intensity, suggesting a time and/or intensity dependent conversion of the signalling pathway from a Ca²⁺/calmodulin-dependent to an AMP-dependent pathway (86; 222). These recent observations provide support for the hypothesis proposed by Ojuka *et al.* (149; 150), however further studies are needed to elucidate the nature of the cross-talk between CaMK II and AMPK during exercise.

MAPKs such as p38 are ubiquitous signalling proteins that can activate transcription factors in response to a variety of growth factors and environmental stresses, including exercise (177). It has been reported that exercise activates p38 and causes it to associate with and activate MEF2A in human skeletal muscle (121; 153; 213; 228; 233). Some reports indicate that p38 activation may signal to increase GLUT4 expression. For example, Montessuit *et al.* (132) demonstrated that expression of a constitutively active form of the p38 upstream regulator, MAPK Kinase (MKK) 6, results in increased *Glut4* promoter activity in neonatal rat cardiomyocytes. Furthermore they showed that p38 is required for IGF-1-induced GLUT4 expression in adult rat cardiomyocytes. In other cases however where p38 was activated in muscle cells by over-expression of p38 γ or by expression of constitutively active MKK6/3, GLUT4 expression was reduced (46; 68). Clearly more research is required to understand whether p38 plays a role in regulating GLUT4 expression in skeletal muscle during exercise.

Calcineurin is a phosphatase, which is activated by calmodulin in response to low amplitude, prolonged calcium transients and mediates the conversion of fast to slow twitch muscle fibres after extended periods of exercise training (23; 139; 226). The observation that GLUT4 levels are elevated in transgenic mice that over-express a constitutively active calcineurin, led to the notion that calcineurin might also be responsible for increasing GLUT4 expression after exercise (175). Garcia Roves *et al.* (50) however, used rats that were injected with the calcineurin inhibitor, cyclosporin, to show that calcineurin was not necessary for exercise

to increase GLUT4 expression. The use of a metabolic inhibitor in these experiments does not exclude the possibility that other signals that are also activated by exercise may have overcompensated for the lack of calcineurin activity. It is also possible that calcineurin may play a more significant role in response to muscle contractions that initiate low amplitude, prolonged calcium transients. Therefore, although calcineurin may not be necessary for the exercise-induced increases in GLUT4 expression, it is still unclear whether it is capable of increasing GLUT4 expression in response to extended periods of low intensity muscle contraction.

1.10 Summary

There is ample evidence that exercise improves whole body insulin sensitivity by increasing the abundance of GLUT4 in skeletal muscle (24; 25; 84). The increase in skeletal muscle GLUT4 expression after a bout of exercise is regulated by MEF2A/MEF2D and GEF transcription factors, which bind to their binding domains in the *Glut4* promoter *in vitro* (122). MEF2 transcriptional activity is repressed by HDACs which SUMOylate MEF2 and deacetylate histones in gene promoters (113; 235). In contrast, HATs increase MEF2 binding to DNA and transcriptional activity by acetylating MEF2 and histones in MEF2-regulated gene promoters, respectively (115; 125). CaMK removes the repression of MEF2 by HDACs (124) and has been shown to be necessary for the acetylation of histones in the promoters of some muscle genes that are regulated by MEF2 (181). Experiments in cultured myotubes have also shown that CaMK activation induces an increase in GLUT4 protein content (150). However, the role that CaMK plays in GLUT4 expression in response to exercise has not been investigated. Furthermore, the pattern of histone acetylation within the *Glut4* promoter and the influence of exercise or CaMK activity on this acetylation is not known. The role of CaMK II activity in exercise-induced GLUT4 expression therefore requires further investigation and is the focus of this thesis.

CHAPTER TWO

The main objectives of this project

The general purpose of this research was to elucidate some of the molecular mechanisms whereby exercise training increases the expression of GLUT4 in skeletal muscle. Understanding these mechanisms is important because they may reveal novel targets for pharmaceuticals to treat insulin resistance and type II diabetes in patients who are unable to exercise because of ill health.

As discussed in section 1.5, MEF2 transcription factors have been reported to regulate GLUT4 expression in response to exercise. Using EMSA, it has been demonstrated that when muscle nuclear extracts are incubated *in vitro* with an oligonucleotide containing the MEF2 *cis*-element of the *Glut4* promoter, there was more binding of MEF2A/MEF2D to the element when the muscle were taken from humans that had exercised, compared to sedentary controls (122; 184). These observations have been presented as evidence that exercise increases the binding of these factors to the *Glut4* promoter to enhance GLUT4 expression. However, it is uncertain whether results from these *in vitro* binding assays are a true reflection of their binding to the *Glut4* promoter *in vivo*. Unlike in EMSA, where binding domains on DNA are freely accessible to transcription factors, *in vivo* DNA is bound to histone proteins through electrostatic forces, and these interactions often limit transcription factor access to their binding sites (9; 102). In light of the marked differences in environmental conditions that exist when transcription factors bind to their *cis*-elements *in vitro* and when they bind *in vivo*, the first purpose of this thesis was to re-evaluate the binding of MEF2A to its binding site on the *Glut4* promoter using an *in vivo* binding assay and to determine the effect of exercise on this association. A rat model of moderate intensity exercise that involves intermittent swimming was developed and chromatin immunoprecipitation (ChIP) assays were optimised for rat muscles (Chapter 3). ChIP assays confirmed that the exercise protocol, which increases GLUT4 expression in skeletal muscle, also increases the binding of MEF2A to its binding domain in the *Glut4* promoter, *in vivo* (Chapter 4).

The second purpose of this thesis was to investigate which signals increase GLUT4 expression and MEF2A binding to the *Glut4* promoter in response to exercise. As discussed in section 1.6 and 1.7, CaMK II is activated in human skeletal muscle during exercise (173; 174) and increases MEF2 transcriptional activity (113). Ojuka *et al.* (150) provided indirect evidence that

CaMK activation was necessary for caffeine-induced GLUT4 expression in L6 myotubes (150). The second aim of this project therefore was to determine if the increase in GLUT4 expression and MEF2A binding to the *Glut4* promoter that was observed after exercise, was mediated by CaMK. When CaMK II activity was inhibited in rats by injection with KN93 prior to exercise, the exercise-induced increase in MEF2A that was bound to the *Glut4* promoter was blocked and the increase in GLUT4 protein expression was significantly reduced (Chapter 5).

The third purpose of this thesis was to explore the mechanisms by which CaMK activation increases GLUT4 expression and the binding of MEF2A to the *Glut4* promoter. There is evidence that CaMK influences the acetylation of histones near MEF2 binding sites in the promoters of muscle genes: In L6 myotubes that were induced to differentiate by incubation with Arg-vasopressin, it was found that CaMK was necessary for a) the increased binding of MEF2A to its site in the MCK and myogenin promoters, b) for the hyper-acetylation of histones in these promoters and c) for enhanced MCK and myogenin protein expression (181). Because CaMK is activated by exercise (173; 174) this thesis therefore tested the hypotheses that a) exercise would induce hyper-acetylation of histones within the *Glut4* promoter and b) inhibition of CaMK II activity would prevent this increase. Using ChIP assays on muscles from rats it was found that exercise increased in the acetylation of histone H3 at the MEF2 binding site in the *Glut4* promoter, whereas inhibition of CaMK activity by KN93 markedly reduced this acetylation (Chapter 5).

Using KN93 to inhibit CaMK activity in rats revealed that CaMK activation is necessary for the increase in GLUT4 expression and MEF2A binding to the *Glut4* promoter in response to exercise. A fourth purpose of this thesis was to test whether CaMK activation is sufficient for these responses. Recombinant adenoviruses that contain a constitutively active (CA) or dominant negative (DN) human CaMK IV gene were therefore produced (Chapter 6) and the recombinant CaMK IV proteins were expressed in C2C12 myotubes. The amount of GLUT4 mRNA and protein and *Glut4* promoter-bound MEF2A was higher in cells expressing CA CaMK IV compared to those expressing DN CaMK IV providing direct evidence that CaMK activity is sufficient to increase GLUT4 expression in mouse skeletal muscle (Chapter 7).

In summary, the main objectives of the current investigation were:

1. To evaluate the *in vivo* binding of MEF2A to the *Glut4* promoter in skeletal muscle after exercise.

2. To investigate whether CaMK activity is necessary for the exercise-induced increases in GLUT4 expression and MEF2A binding to the *Glut4* promoter in skeletal muscle.
3. To investigate the effect of CaMK inhibition on the acetylation of histones within the *Glut4* promoter in skeletal muscle after exercise.
4. To determine whether CaMK activation was sufficient to increase MEF2A binding to the *Glut4* promoter and GLUT4 expression in skeletal muscle.

University of Cape Town

CHAPTER THREE

Establishment of exercise and assay protocols

3.1 Introduction

A rat model of moderate-intensity intermittent exercise was used to evaluate the mechanisms whereby exercise increases GLUT4 expression in skeletal muscle. In one set of experiments to establish the time course of CaMK II activation, *in vivo* MEF2A binding to the *Glut4* promoter and changes in GLUT4 mRNA and protein levels, muscles were isolated from rats at various time points after they had completed a 5 x 17 minute swim carrying a load of 4% body weight. In a second series of experiments to investigate the role of CaMK II in these responses to exercise, CaMK activity was inhibited with KN93 prior to swimming. This chapter describes the development of the swimming protocol (section 3.3) and the methods used for these investigations (sections 3.5 - 3.14).

3.2 Source of materials used

Wistar rats were from the University of Cape Town animal unit (Cape Town, South Africa). KN93 was purchased from Calbiochem (San Diego, CA). Pentobarbital Sodium (Sagatal) was from May and Baker (Port Elizabeth, South Africa). The suppliers of the primary antibodies for western blots are described in Table 3.3. Polyclonal horseradish peroxidase (HRP) conjugated goat anti-rabbit and rabbit anti-mouse secondary antibodies were purchased from Dako (Carpinteria, CA). Fluorochrome donkey anti-rabbit Cy3 antibody was purchased from Dianova (Hamburg, Germany). ChIP and CaMK II assay kits were from Upstate Cell Signalling Solutions (Charlottesville, VA) and Redivue [γ 32 P] ATP was from AEC-Amersham (Cape Town, South Africa). Primers (Table 3.4) were designed according to section 3.8.5 and were synthesized by INQABA Biotechnological Industries (Cape Town, South Africa). Ribosomal S12 (RS12) primers were a generous gift from Prof. Sue Kidson's laboratory, University of Cape Town, South Africa. Tri reagent was from Ambion Inc. (Austin, TX) and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase, RNasin and RNase free DNase I was from Promega (Madison, WI). Real Time PCR reagents were from Qiagen (Valencia, CA). SuperTherm Taq DNA polymerase was purchased from Medox Biotech (Chennai, India). Complete protease inhibitors

were from Roche Diagnostics (Randburg, South Africa). UN-SCAN it software was from Silk Scientific (Orem, USA). All other chemicals and materials were purchased from Sigma (St Louis, MA).

3.3 Rat exercise protocol

3.3.1 Animal care. Five week old male Wistar rats that weighed 200-250 g were used for these studies. All procedures involving animals were approved by the Animal Research Ethics Committee (AEC) of the University of Cape Town. Rats, which were housed 4-6 per cage in a room maintained between 21 and 24°C with a 12-hour light/dark cycle, were fed standard rat chow and water, *ad libitum*. Their welfare and weights were checked daily and they were not left unattended while swimming.

3.3.2 Exercise protocol. The swimming protocol used in this thesis is similar to the protocol described previously by Terada *et al.* (1993). Rats were swum separately in cylindrical barrels with a surface area of ~1200 cm² filled to a depth of 50 cm with clean tap water maintained at 35°C. Initially, all rats were familiarized to intermittent swimming with a weight attached to the base of their tails by gradually increasing the weight and the number of bouts, as illustrated in Table 3.1 (days 5-8). The weights did not cause distress to the rats and did not impede swimming action. In all swim sessions, rats rested for 3 minutes between bouts. They were dried with towels and kept warm after each bout and before being returned to their cages. By the end of the familiarization period (day 8), rats could complete 3 bouts each lasting 17 minutes carrying a load equivalent to 4% body weight. After the familiarisation period rats were rested for 6 days to eliminate any adaptation that may have resulted from the training. On the last day of the rest period (day 14), rats were fasted overnight and were used in 1 of 2 sets of experiments the following day.

In the first set of experiments, the 'exercise' group, which was chosen randomly, underwent a final swimming session consisting of 5 x 17 minutes carrying a load equivalent to 4% body weight. After the swim, rats were killed immediately (0h) or dried and returned to their cages and killed at 0, 0.5, 2, 6 or 18 hours later. In all cases rats were anaesthetized with an intraperitoneal (i.p.) injection of ~ 50 mg.kg⁻¹ pentobarbital sodium and triceps muscles were dissected out, immediately frozen in liquid nitrogen and stored at -80°C. With particular reference to the rats that were killed immediately after the swim, it should be noted that the lag time

between the end of the swim and the freezing of triceps muscle was about 10-15 minutes. Rats that were not exercised were used as controls. All swim sessions took place between 8 and 11 am to avoid diurnal variations.

Table 3.1. The rat handling, familiarisation and swimming protocol for the first series of experiments to measure the time-course of GLUT4 expression after exercise.

Day	Intervention	
	CONTROL RATS	EXERCISED RATS
1	Rats were received, weighed and placed in cages.	
2-4	Rats were familiarised to handling and health was monitored.	
5	2 x 17 minute swim with a 3 minute rest between bouts	
6	3 x 17 minute swim with a 3 minute rest between bouts	
7	4 x 17 minute swim with a 3 minute rest between bouts	
8	3 x 17 minute swim with a 3 minute rest between bouts with load of 4% body weight	
9-14	Rats kept in cages. On day 14 rats were fasted overnight.	
15	Rats had a load of 4% body weight attached to their tails and were returned to their cages	Rats had a load of 4% body weight attached to their tails and completed a 5 x 17 minute swim with a 3 minute rest between bouts
	Rats were anaesthetised and triceps muscle dissected out at 0, 0.5, 2, 6 or 18 hours post exercise.	

In the second series of experiments outlined in Table 3.2, rats were familiarised with swimming, rested for 6 days and fasted on the night before the experiment as before. On the day of the experiment they were randomly divided into 3 groups: The first and second groups were given an i.p. injection of 5 mg.kg⁻¹ KN93 or an equal volume of vehicle (saline) respectively, 30 minutes before beginning the final exercise session consisting of 5 x 17 minutes of swimming with a tail load equivalent to 4% body weight. This dose of KN93 has been previously reported to normalise elevated levels of CaMK II activity in rats (11). Rats were anaesthetised at 0, 6 or 18 hours after the final bout and triceps muscle were isolated and frozen immediately as described above. In experiments where GLUT4 protein was to be measured, rats were injected with KN93

and exercised on two consecutive days and muscles were obtained 18 hours after the last exercise bout to ensure that a more robust increase in GLUT4 protein was observed. A third group of rats that formed the control group, also had weights attached to their tails and were injected with saline but kept in their cages while rats in the other groups performed the final swim. Muscles from these rats were isolated at similar time points to the exercised rats.

Table 3.2. The rat swimming protocol for the second series of experiments to measure the effect of CaMK inhibition on exercise-induced GLUT4 expression.

Day	Intervention		
	CONTROL	EXERCISED	EXERCISED + KN93
1-14	Rats were familiarised (day 5-8), rested (day 9-14) and fasted the night before the experiment (on day 14) as per Table 3.1.		
15	Rats were injected with saline.	Rats were injected with saline.	Rats were injected with KN93.
	Rats had a load of 4% body weight attached to their tails and were returned to their cages for 30 minutes.		
	Rats remained in their cages	Rats completed a 5 x 17 minute swim with a 3 minute rest between bouts	Rats completed a 5 x 17 minute swim with a 3 minute rest between bouts
	Rats were anaesthetised and triceps muscle dissected out at 0 or 6 hours after the time of the last bout of swimming.		
16	An additional set of rats that were not killed, repeated day 15 and were anaesthetised and triceps muscle dissected out at 18 hours after the time of the last bout of swimming.		

3.4 Justification for the rat swimming protocol

3.4.1 The rat model. According to the NIH Meeting on Rat Model Priorities (May 1999) (<http://www.nhlbi.nih.gov/resources/docs/ratmtg.htm>), rats are considered the most appropriate experimental model of human disease and are a useful tool to study the relationship between genes and biological function in humans. Additionally, the rat genome has been entirely sequenced and vast amounts of data exist on rat physiology, behaviour, biochemistry, genetics and pharmacology.

3.4.2 Swimming protocol: Earlier studies have shown that the swimming protocol used in this study (see section 3.4.2) was as effective at increasing GLUT4 expression as the traditional protocol which consisted of 6 hours of swimming (193), but is more time efficient. Familiarising the rats to the swimming protocol reduced anxiety and ensured that the observed changes in the measured variables were due to exercise and not due to the stress.

3.4.3 Rat muscle: Triceps muscle was used in this study because they are large enough to provide sufficient sample for analysis (~250 mg from each fore limb) and adapt to exercise with a robust increase in GLUT4 mRNA and protein content (50).

3.4.4 Time of dissection: Previous experiments have shown that GLUT4 transcription rate and mRNA levels increase within 1.5-6 hours after a single bout of exercise whereas GLUT4 protein content increases within 3-18 hours after exercise and accumulates with repeated daily bouts (section 1.4). Muscles were therefore dissected from rats between 0 and 18 hours after a single swimming session to measure CaMK II activation, the acetylation of histones within the *Glut4* promoter, MEF2A binding to the *Glut4* promoter and GLUT4 mRNA levels. To measure GLUT4 protein, rats in the experiments that used KN93 were exercised on 2 consecutive days and muscles were isolated 18 hours after the last bout.

3.4.5 Rat strain: Wistar rats are not aggressive, easy to handle and were readily available. Previous experiments in our and other laboratories have shown that Wistar rats are capable of completing the swimming protocol and readily adapt to swimming with improvements in glucose transport and GLUT4 content in skeletal muscle (50).

3.4.6 Rat gender: Male rats were used to avoid factors introduced during the oestrus cycle of female rats, such as increased levels of physical activity, reduced food and water intake and body weight loss (37). All these factors could potentially confound the results.

3.4.7 Rat age: In pilot studies in our laboratory, rats younger than 4 weeks (weighing 100-150 g) were unable to comfortably complete the familiarisation swimming protocol. Rats beginning the protocol at 5 weeks of age (weighing 200-250 g), could comfortably complete the swimming sessions and showed a robust increase in muscle GLUT4 expression. Older rats were not used because they become more aggressive and are more expensive to maintain.

3.4.8 Rat number: 4-8 rats per experimental group were used in these studies to provide sufficient statistical power to detect changes in the dependent variables.

3.5 Tissue homogenisation and protein concentration determination

Approximately 50 mg of frozen muscle was ground to a powder in liquid nitrogen, transferred to an eppendorf tube and mixed with 1 ml of an appropriate buffer. HES buffer (20 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES); 1 mM ethylene diamine tetra-acetic acid (EDTA); 250 mM Sucrose with 1 x Complete protease inhibitors (Roche)) was used for GLUT4, MEF2A and α -tubulin western blots. For western blots to detect phosphorylated residues on proteins, a 'Phospho' buffer (50 mM Tris-hydrochloric acid (HCl), pH 7.4; 150 mM sodium chloride (NaCl); 1 mM EDTA; 20 mM sodium fluoride (NaF); 10 mM sodium pyrophosphate ($\text{Na}_4\text{P}_2\text{O}_7$); 100 nM ocadaic acid; 1 mM sodium orthovanadate (Na_3VO_4); 0.2 mM phenylmethylsulphonic acid (PMSF); 1% Nonidet P-40 (NP-40); 1 x Complete protease cocktail (Roche)) was used. For CaMK II activity assays, the buffer consisted of 50 mM Tris-HCl, pH 7.5; 250 mM sucrose; 1 mM EDTA; 1 mM ethylene glycol tetra-acetic acid (EGTA); 1 mM dithiothreitol (DTT); 20 mM NaF; 5 mM $\text{Na}_4\text{P}_2\text{O}_7$; 10% glycerol, 1 mM PMSF, 1 mM Na_3VO_4 ; 1% NP-40; and 1x Complete protease inhibitors (Roche). The samples were homogenised on ice for 30 seconds using a glass homogeniser attached to drill press at slow speed. Muscle homogenates were centrifuged at 8000 x g for 10 minutes and the protein concentration of the supernatants determined using the Bradford assay as follows: ~5 μl of protein sample was incubated with 1 ml of Bradford reagent (0.02% coomassie brilliant blue G250; 4.75% ethanol; 8.5% phosphoric acid) for 5 minutes and the absorbance at 595 nm was measured. Concentrations of unknown samples were calculated based on a standard curve generated using a bovine serum albumin (BSA) solution of known concentration.

3.6 Western Blots

3.6.1 Western blots: The protein homogenates described in section 3.5 were solubilised in Laemmli sample buffer (250 mM Tris-HCl, pH 6.8; 2% SDS, 10% glycerol; 0.01% bromophenol blue, 5% β -mercaptoethanol), incubated at 95°C for 5 minutes and used immediately for western blots or stored at -80°C. For GLUT4 western blots, β -mercaptoethanol was omitted from the sample buffer and proteins were not heated. A quantity of protein (described in Table 3.3) was

loaded onto a 10% SDS polyacrylamide gel and run at 100 V for ~1.5 hours. Five μl of a protein standard (Precision Plus, Biorad) was included in one lane. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham) using a transfer apparatus (Biorad) at 200 mA for 1.5 hours in a buffer containing 25 mM Tris-HCl; 192 mM glycine; and 15% methanol. Membranes were rinsed in phosphate buffered saline containing 0.1% Tween 20 (PBST) and blocked in PBST containing 5% non-fat milk powder for 1 hour at room temperature. Membranes were rinsed again and incubated in primary antibodies. The antibody source, antibody concentrations and incubation buffers, times and temperatures for the various western blots are shown in Table 3.3. The membranes were washed for 3 x 10 minutes in PBST and incubated with appropriate HRP-conjugated secondary antibodies, diluted 1: 10 000 in PBST, for 1 hour at room temperature. Membranes were washed as before, blotted dry and incubated in enhanced chemiluminescence (ECL) detection solution (Amersham) for 1 minute and exposed to radiographic film for 1-3 minutes. Films were developed using regular photographic chemicals and were scanned at 300 dpi in 8 bit greyscale mode. The intensities of the bands were quantified using UN-SCAN-IT gel 6.1 software (Silk Scientific). Signal intensities, representing the protein content, were normalised to α -tubulin and expressed relative to a control in each blot.

3.6.2 Stripping of antibodies from membranes: Membranes that had previously been used in western blots were washed briefly in PBST and incubated for 30 minutes in pre-warmed buffer (0.5 M Tris HCl, pH 6.8; 10% SDS; 0.8% β -mercaptoethanol) at 50°C. Membranes were washed again for 10 minutes in PBST, blocked and re-probed for α -tubulin as described in section 3.6.1.

3.7 Measurement of muscle glycogen content

Glycogen content was calculated by measuring the glucose concentration of hydrolysed glycogen that was extracted from muscle samples as described by Passonneau and Lauderdale (156). Briefly, ~20 mg of frozen muscle was weighed and digested in 200 μl of cold 40% KOH in pre-weighed tubes by heating for 30 minutes at 95°C. Once cooled, 800 μl of absolute ethanol was added and the samples were stored at 4°C overnight to precipitate the glycogen. The samples were centrifuged for 15 minutes at 13 000 x g and the pellets were resuspended in 2 N HCl and heated for 3 hours at 95°C to hydrolyse the glycogen to glucose equivalents. Samples were centrifuged for 3 minutes and 100 μl of 0.2 M Tris-HCl (pH 7.5) and 150 μl of 2 M Sodium Hydroxide (NaOH) was added to the supernatants to neutralise the pH of the samples. Assay samples were

weighed in their tubes and the glucose concentration was determined using a glucose analyser (Beckman). Glycogen content was calculated using the following formula:

$$\text{muscle glycogen (mmol.kg}^{-1}\text{)} = \frac{[\text{glucose}] (\text{mg.dl}^{-1}) \times \text{assay sample weight (mg)} - \text{tube weight (mg)}}{18 \times \text{muscle weight (mg)}}$$

Table 3.3. Incubation conditions for primary and secondary antibodies and the amount of protein used from rat and C2C12 muscle homogenates in western blots.

Primary antibody (catalogue) supplier, address	Amount in μg and (type) of protein homogenate used	Primary antibody dilution	Primary antibody buffer	Secondary antibody
GLUT4, Donated by Mike Mueckler, Washington University School of Medicine.	8-10 (rat) 50-70 (C2C12)	1:5 000	PBST + 1% MP	rabbit
MEF2 H300 (sc-10794), Santa Cruz Biotechnology, Santa Cruz, CA.	30 (rat) 50 (C2C12)	1:500	PBST + 1% MP	rabbit
MEF2D (M62720). BD Transduction Laboratories, Lexington, KY.	30 (rat) 50 (C2C12)	1: 2 500	PBST + 1% MP	mouse
α -tubulin (sc-8035). Santa Cruz Biotechnology, Santa Cruz, CA.	30-70 (rat, C2C12)	1:500	PBST + 1% MP	mouse
PGC-1 H300 (sc-13067), Santa Cruz Biotechnology, Santa Cruz, CA.	30 (rat) 50 (C2C12)	1:500	PBST + 1% MP	rabbit
Phospho-CREB Ser133 (600-401-270), Rockland, Gilbertsville, PA	40 (rat) 70 (C2C12)	1:500	PBST + 5% BSA	rabbit
CREB-1 (100-401-195), Rockland, Gilbertsville, PA.	40 (rat) 70 (C2C12)	1:1 000	PBST + 1% MP	rabbit
p38 (# 9212), Cell Signalling Technology, Danvers, MA.	30 (C2C12)	1:1 000	PBST + 5% BSA	rabbit
FLAG M2 (F3165), Sigma St Louis, MA	50 (C2C12)	1:1 000	PBST + 1% MP	mouse
Phospho-CaMK II Thr 286 (#3361), Cell Signalling Technology, Danvers, MA.	30 (rat)	1:1 000	PBST + 5% BSA	rabbit
CaMK II (ab5683), Abcam, Cambridge, MA.	30 (rat)	1:1 000	PBST + 1% MP	rabbit
Acetyl histone H3 Lys9/Lys14 (# 9677), Cell Signalling Technology, Danvers, MA.	Used for ChIP			

Membranes were incubated with the primary antibodies at 4°C overnight and with HRP-conjugated secondary antibodies, diluted 1:10 000 in phosphate buffered saline with 1% Tween 20 (PBST) for 1 hour at 25°C. C2C12 = C2C12 homogenate, rat = rat muscle homogenate, MP = fat free milk powder, BSA = bovine serum albumin.

3.8 Analysis of mRNA

3.8.1 RNA extraction: Total RNA was isolated from muscles using Tri reagent based on the manufacturers instructions (Ambion). One hundred mg of frozen muscle was ground to a powder in liquid nitrogen and combined with 1 ml of Tri reagent. Samples were vortexed briefly, incubated at room temperature for 15 minutes and the cellular debris pelleted and removed by centrifugation. Two hundred μ l of chloroform was added to the supernatants and the samples were vortexed for 15 seconds, incubated for 15 minutes and centrifuged again. The aqueous phase was transferred to a new tube and RNA was precipitated overnight at -20°C with a volume of isopropanol that equalled the aqueous phase. The RNA was pelleted by centrifugation for 15 minutes at $13\ 000 \times g$, washed in 75% ethanol, air dried and resuspended in 20 μ l nuclease-free water.

3.8.2 Determination of RNA concentration and integrity: The concentration and optical density at 260 and 280 nm of the RNA was measured in triplicate using 1 μ l of RNA in a Nanodrop ND 1000 spectrophotometer (Nanodrop Technology). RNA extracts were accepted as pure from protein contamination when the 260/280 nm ratio was between 1.9 and 2.1. The integrity of the RNA was checked as follows: Three μ g of RNA was diluted 2-fold in buffer containing 6% glycerol, 1% MOPS, 2 M formaldehyde, 0.5% formamide, and bromophenol blue. Samples were heated at 55°C for 15 minutes and separated by electrophoresis on a 1% agarose gel containing 1X MOPS, 0.66 M formaldehyde and 35 μ g Ethidium Bromide. RNA was visualised under UV light and deemed un-degraded if the 28S and 18S ribosomal bands were observed intact.

3.8.3 cDNA synthesis: One μ g of RNA was combined with 0.5 μ g of a 22-mer oligo dT primer and was denatured at 70°C for 5 minutes. Genomic DNA was eliminated from the samples by digestion with 1 U of DNase 1 (Promega) for 90 minutes at 37°C . The DNase 1 enzyme was deactivated by incubating at 75°C for 5 minutes. cDNA was synthesized from the denatured RNA using 200 U of M-MLV reverse transcriptase (Promega) at 42°C for 1 hour in a reaction mix containing 2 mM deoxyribonucleotide triphosphates (dNTPs), 2.5 mM magnesium chloride (MgCl_2), 20 U RNAsin (Promega) and 1 X reverse transcriptase buffer (Promega). Aliquots of cDNA were stored at -20°C .

3.8.4 Real Time quantitative PCR: Real Time quantitative PCR (RTqPCR) was performed to measure relative GLUT4 mRNA content using a Roche Light Cycler PCR machine, SYBR

green PCR plus reagents (Roche) and primers that are described in Table 3.4. Ten μl PCR reactions containing 1 μl of cDNA; 1 x SYBR green enzyme mixture and 0.5 μM of forward and reverse primers were performed in triplicate using the following conditions: 1 cycle at 94°C for 10 minutes, followed by 35 cycles at 94°C for 10 seconds, 62°C for 10 seconds, and 72°C for 15 seconds. Fluorescence data (indicating the amount of double stranded PCR product) was captured after each cycle. The relative GLUT4 mRNA concentration was calculated based on the cycle number that a threshold quantity of PCR product was obtained (threshold cycle - Ct). Ct values for GLUT4 mRNA were normalised to values from glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal protein S12 (RS12) internal house keeping genes and expressed relative to a control in each experiment, according to the method described by Livak *et al.* (112). Data using the GAPDH housekeeping gene are presented in Chapter 4, 5 and 7. Data using the RS12 house keeping gene were used for confirmation purposes and are not shown in the results of this thesis. At the end of the PCR, a melting curve with continuous fluorescence data acquisition was performed by gradually increasing the temperature of the RNA to confirm that single melting peaks (indicating single PCR products) were obtained for each reaction. Samples were also separated on a 1% agarose gel by electrophoresis and stained with ethidium bromide to ensure that single bands were observed.

3.8.5 Primer Design: Oligonucleotides consisting of 18-22 bp were designed using Primer 3 software from DNA sequences obtained from an online database (Pubmed, www.ncbi.nlm.nih.gov). Primers with the least potential to form inter- or intra- primer dimers and hairpins were selected and primers with excessive single nucleotide runs were avoided. Wherever possible, primers with a GC content of 50-70% and a melting temperature of 60-70°C were used. Primers for real time PCR were designed to span an exon-exon boundary to avoid amplification of genomic DNA. Where exon-exon boundaries were not published, exon-intron segments were determined by aligning cDNA sequences with genomic DNA sequences obtained from the Pubmed database. Primers for ChIP assays were designed to span binding sites in the 5' upstream promoters of genes. As a negative control for ChIP assays, primers were designed which amplify a region in the gene that does not contain the binding element under investigation. When promoter regions were not available, the 5' upstream sequence was identified by aligning cDNA sequences with genomic DNA sequences. Primer sequences and the size of the products amplified by PCR are described in Table 3.4.

Table 3.4. Primers and the size of products generated in PCRs using mouse (M), rat (R) or human (H) cDNA or genomic DNA.

Name assigned to the primer pair	Template DNA used for primer design (primer use)	Amplicon size in bp using cDNA (species)	Amplicon size in bp using genomic DNA (species)	Forward (F) and reverse (R) primer sequences
GLUT4 cDNA	Rat and mouse GLUT4 cDNA (GLUT4 mRNA)	135 (M) 130 (R)	243 (M)	F: 5'-GCAGCGAGTGACTGGAACA-3' R: 5'-CCAGCCACGTTGCATTGTAG-3'
PGC-1 α cDNA	Rat and mouse PGC-1 α cDNA (PGC-1 α mRNA)	142 (R) 147 (M)	626 (R) 651 (M)	F: 5'-AAGGATGCGCTCTCGTTC-3' R: 5'-GGTGTCTGTAGTGGCTTG-3'
RS12 cDNA	Rat and mouse RS12 cDNA (house keeping for real time PCR)	380 (R) 380 (M)		F: 5'-GGAAGGCATAGCTGCTGGAGGTGT-3' R: 5'-CGATGACATCCTTGGCCTGAG-3'
rGAPDH cDNA	Rat GAPDH cDNA (house keeping for real time PCR)	183 (R)		F: 5'-GAACATCATCCCTGCATCC-3' R: 5'-CCTGCTTCACCACCTTCTT-3'
mGAPDH cDNA	Mouse GAPDH cDNA (house keeping for real time PCR)	100 (M)		F: 5'-GGCTCTCCAGAACATCATCC-3' R: 5'-GCCTGCTTCACCACCTTC-3'
r <i>Glut4</i> promoter (+ve primers)	Rat <i>Glut4</i> promoter (MEF2A and Acetyl histone H3 ChIP)	N P (R) N P (M)	350 (R)	F 5'-GACACGGTTCTCAGACACAGC-3' R 5'-CTGAGAGGTGGAAGAGGAGG-3'
m <i>Glut4</i> promoter (+ve primers)	Mouse <i>Glut4</i> promoter (MEF2A and Acetyl histone H3 ChIP assay)	N P (R) N P (M)	268 (M)	F 5'-CAGGCATGGTCTCCAGATACAC-3' R 5'-GGTAACTCCAGCAGGATGACA-3'
r <i>Glut4</i> CON (-ve primers)	Rat <i>Glut4</i> gene (-ve control for MEF2A ChIP assay)	285 (R)	285 (R)	F 5'-GACGGACACCTTCTCTTTC-3' R 5'-CCACAGCCTAGCCACAACAC-3'
m <i>Glut4</i> CON (-ve primers)	Mouse <i>Glut4</i> gene (-ve control for MEF2A ChIP assay)	315 (M)	315 (M)	F 5'-CCAACAGCTCTCAGGCATCAA-3' R 5'-CCATTCCACAGGCAAGCAG-3'
hCaMK IV N	Human CaMK IV amino terminal region (identification of recombinant CaMK IV constructs)		200 (H) N P (R, M)	F: 5-AACAGGGATGCGCTGAG-3' R: 5-TGGATGTGAGAGGCGAAG-3'
hCaMK IV C	Human CaMK IV carboxyl terminal region (identification of recombinant CaMK IV constructs)		200 (H) N P (R, M)	F 5'-CCAAGCCGAGTTAAGG-3'; R 5'-TGCTCTCCACAGTCTTC-3'

N P = no product formed in the PCR (the primers were designed not to recognise the specified template)

3.9 Chromatin immunoprecipitation Assays

ChIP assays were performed on rat skeletal muscle as illustrated in Figure 3.1.

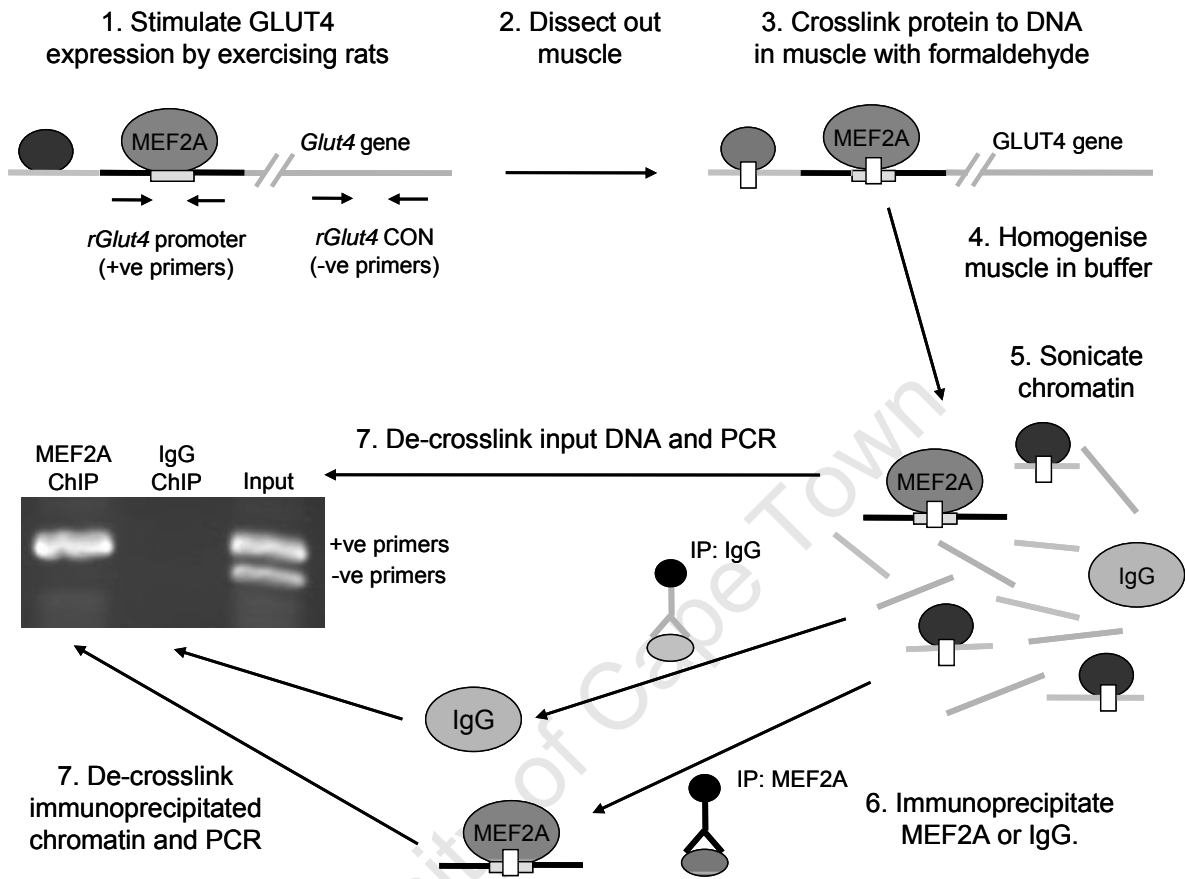


Figure 3.1. The chromatin immunoprecipitation assay to measure MEF2A binding to the *Glut4* promoter. Rats were exercised to stimulate GLUT4 expression and triceps muscles were isolated, ground in liquid nitrogen and incubated with formaldehyde to crosslink proteins to DNA. Samples were homogenised and the DNA sheared to less than 1000 bp by sonication. MEF2A or IgG was immunoprecipitated from the samples and the co-immunoprecipitated chromatin was de-crosslinked, purified and used in PCR with primers that amplify the MEF2 binding site in the *Glut4* promoter and primers that amplify a region 5 kb downstream in the *Glut4* gene that does not contain a MEF2 *cis*-element (-ve CON). PCR was also performed on chromatin purified from an aliquot of the sample that did not undergo immunoprecipitation (Input). PCR products were resolved on a 2% agarose gel, stained with ethidium bromide and visualised under UV.

3.9.1 Formaldehyde crosslinking and sonication of chromatin: One hundred mg of frozen muscle was ground to a powder in liquid nitrogen and crosslinked in 10 ml of PBS containing 1% formaldehyde for 10 minutes at 25°C with gentle rotation on an ordinary bench top rotator. Crosslinking was stopped by adding glycine to a concentration of 0.125 M for 5 minutes and the

muscle was washed three times with cold PBS. The samples were centrifuged at 2000 x g for 2 minutes and the muscle pellet was lysed on ice in 500 µl SDS lysis buffer (1% SDS; 10 mM EDTA; 50 mM Tris, pH 8.1; 0.5 mM PMSF and 1 x Complete protease inhibitors (Roche)). Homogenates generated from crosslinked samples were sonicated for 0, 4, 8 or 16 x 15 second bursts at 33% of maximum intensity using a Virsonic 60 sonicator (Virtis). The samples were kept on slushy ice at all times and the period between bursts was at least 1 minute to prevent samples from over heating. Samples were centrifuged twice at 13 000 x g for 10 minutes at 4°C and the protein concentration of the supernatants determined. Aliquots of the supernatant were reverse crosslinked by adding 0.2 M NaCl and incubating at 65°C for 6 hours. Chromatin was purified by phenol chloroform extraction as described in section 3.10 and separated on a 1% agarose gel by electrophoresis to confirm sonication efficiency. Figure 3.2 shows that 8 bursts of sonication was sufficient to shear chromatin to fragments of less than 1000 bp, which is suitable for ChIP assays. Samples that were crosslinked for more than 30 minutes resulted in chromatin that was resistant to shearing (data not shown). Interestingly, when the sonicator intensity was set at 100% of maximum, chromatin shearing efficiency was less than when the sonicator was set at 33% of maximum (data not shown). This decrease in efficiency however, is likely attributed to the formation of foam at the higher intensity, which dissipates sonication energy. For all subsequent experiments chromatin was crosslinked by incubating with 1% formaldehyde for 10 minutes at 25°C and sonicating for 8 x 15 second bursts at a sonicator intensity setting of 33% of maximal.

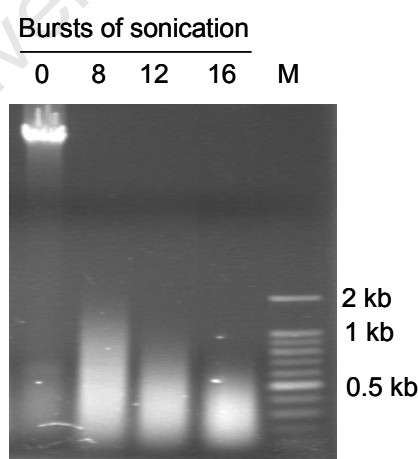


Figure 3.2. Optimising chromatin shearing for the ChIP assay. Muscle was crosslinked for 10 minutes with 1% formaldehyde, homogenised in lysis buffer and sonicated for 0, 8, 12 or 16 x 15 second bursts at 33% of maximum sonicator intensity as described in section 3.9.1. Chromatin was reverse crosslinked, separated on a 1% agarose gel by electrophoresis and stained with ethidium bromide. M = DNA size marker.

To confirm that the protocol described above effectively crosslinked MEF2A to chromatin and that the reverse crosslinking protocol worked, aliquots of crosslinked and de-crosslinked muscle samples were analysed by western blot using a MEF2A antibody. Figure 3.3 shows the presence of high-molecular weight complexes of MEF2A in crosslinked samples and the absence of these complexes in the non-crosslinked or reverse-crosslinked samples. These data demonstrate that the method used for crosslinking MEF2A to DNA was effective and reversible. The presence of higher molecular weight bands in the formaldehyde treated samples also demonstrates that formaldehyde crosslinking does not mask the epitope recognised by the anti MEF2A antibody, indicating that crosslinked samples are suitable for MEF2A ChIP assays.

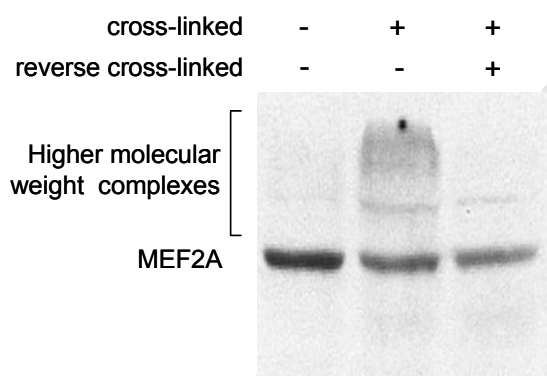


Figure 3.3. The effectiveness of MEF2A crosslinking and de-crosslinking. MEF2A western blot of muscle that was crosslinked with formaldehyde followed by reverse crosslinking as described in section 3.9.1.

3.9.2 Chromatin immunoprecipitation assays. ChIP assays were performed using a Kit from Upstate Cell Signalling Solutions. The protein concentrations of muscle homogenates that were crosslinked and sonicated as described in section 3.9.1, were determined. 100 μ l of sample containing 200 μ g of protein was diluted 10-fold in a buffer (0.01% SDS; 1.1% Triton X-100; 1.2 mM EDTA; 16.7 mM Tris HCl, pH 8.1 and 167 mM NaCl). The samples were pre-cleared of endogenous immunoglobulins by incubating with 40 μ l (~ 80 μ g) salmon sperm DNA/protein A agarose for 30 minutes followed by centrifugation at 1000 x g for 1 minute. The resultant supernatant was referred to as the 'input'. This input sample was incubated with either an antibody against MEF2A (25 μ l) or Histone H3 that is acetylated at Lys 9 and Lys 14 (5 μ l) for 36 hours at 4°C. The immune complexes were precipitated with 60 μ l (~120 μ g) of protein A agarose/ salmon sperm DNA for 1 hour and washed 5 times in kit buffers. To control for non-specific binding of chromatin to the agarose beads, reactions with a non-specific antibody (5 μ l

mouse IgG) and those without any antibody were also run in parallel. To confirm that the anti-MEF2A antibody used in the ChIP assays effectively immunoprecipitated all MEF2A, aliquots of samples that were immunoprecipitated with an anti-MEF2A antibody or without any antibody were used in MEF2A western blots. Figure 3.4 shows the presence of MEF2A in immunoprecipitations that did not contain an antibody and the absence of MEF2A when the anti-MEF2A antibody was included.

Precipitated complexes from ChIP assays were eluted in a buffer consisting of 1% SDS and 0.1 M NaHCO₃ and were reverse crosslinked by adding 0.2 M NaCl and incubating at 65°C for 6 hours. The co-immunoprecipitated DNA was purified by phenol-chloroform extraction and resuspended in 20 µl of nuclease free water as described later in section 3.10. A 350 bp fragment corresponding to nucleotides -284 to -634 of the rat *Glut4* promoter, which contains the MEF2 binding site, was amplified by PCR using primers that are described in Table 3.4 (*rGlut4* promoter, +ve primers) as described in section 3.11. A pair of primers specific to a region approximately 5 kb downstream from the MEF2 site (that does not contain a MEF2 *cis*-element (+4620 to +4903 from the *Glut4* start site) was used as a negative control for non-specific binding of chromatin to the immunoprecipitation antibody (*rGlut4* CON, -ve primers). PCR products were separated on 2% agarose gels, stained with ethidium bromide, photographed and the densities of the bands quantified. Purified DNA from input sample that did not undergo immunoprecipitation was PCR amplified and used to control that equal amounts of sample were used for each immunoprecipitation.



Figure 3.4. MEF2A is immunoprecipitated in ChIP assays using the anti-MEF2A antibody. MEF2A western blot of crosslinked muscle homogenates that were immunoprecipitated with (+) or without (-) an anti-MEF2A antibody as described in section 3.9.2.

3.9.3 Determination of the sensitivity and specificity of the ChIP assay: ChIP assays were performed on muscle homogenates using an anti-MEF2A, anti-acetyl Histone H3 or anti-IgG antibody or with no antibody as described in section 3.9.2. Immunoprecipitated chromatin was

purified and used in PCR reactions containing primers spanning the MEF2 binding site in the *Glut4* promoter (+ve primers). Figure 3.5A shows that PCR products were detected when the anti-MEF2A (a) and anti-acetyl Histone H3 (d) antibodies were used, indicating that the ChIP assays could detect MEF2A bound to the *Glut4* promoter and the acetylation of Histone H3 at this site. When either an anti-IgG antibody was used (c), or when the antibody was excluded from the assay (b), negligible PCR products were obtained, which ensured that non-specific precipitation of chromatin was negligible. When primers that span a region 5 kb downstream from the *Glut4* promoter that does not contain a MEF2 *cis*-element (-ve primers) were substituted for the primers that amplify the *Glut4* promoter, no PCR products were detected when using DNA from MEF2A ChIP assays (a). This ensures that the assay is sensitive to within 5 kb of the MEF2 binding site in the *Glut4* promoter and controls for non specific precipitation of chromatin. In PCR reactions using MEF2A ChIP DNA as template, increasing the PCR cycle number (Figure 3.5B) or DNA template concentration (Figure 3.5C), resulted in more abundant product, which ensured that the PCR was in the exponential phase of amplification. Once satisfied that the conditions for the ChIP assay were optimised, MEF2A ChIP assays were performed using increasing amounts of crosslinked muscle sample. Figure 3.5D shows that when increasing amounts of sample were used for ChIP, as confirmed by PCRs of the purified input DNA, there was a corresponding increase in co-immunoprecipitated DNA detected by PCR. Collectively these experiments ensure that the ChIP assays specifically assess MEF2A binding and histone H3 acetylation at the MEF2 binding site in the *Glut4* promoter.

3.10 Phenol chloroform extraction and ethanol precipitation of DNA

Co-immunoprecipitated DNA from ChIP assays and DNA from input samples was purified from protein homogenates by adding an equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1), mixing for 5 minutes and centrifuging at 13 000 x g for 2 minutes. The aqueous phase was transferred to a new tube and a volume of chloroform: isoamyl alcohol (24: 1) that equalled the aqueous phase was added to it. The samples were mixed and centrifuged as before. The aqueous phase was transferred to a new tube and DNA was precipitated by adding ethanol to a concentration of 70% and sodium acetate to a concentration of 0.3 M. The samples were incubated at -20°C overnight and the DNA was pelleted by centrifugation at 13 000 x g for 15 minutes. The pellets were washed in 70% ethanol, re-centrifuged at 7000 x g for 5 minutes, air-dried and re-suspended in 20 µl nuclease free water.

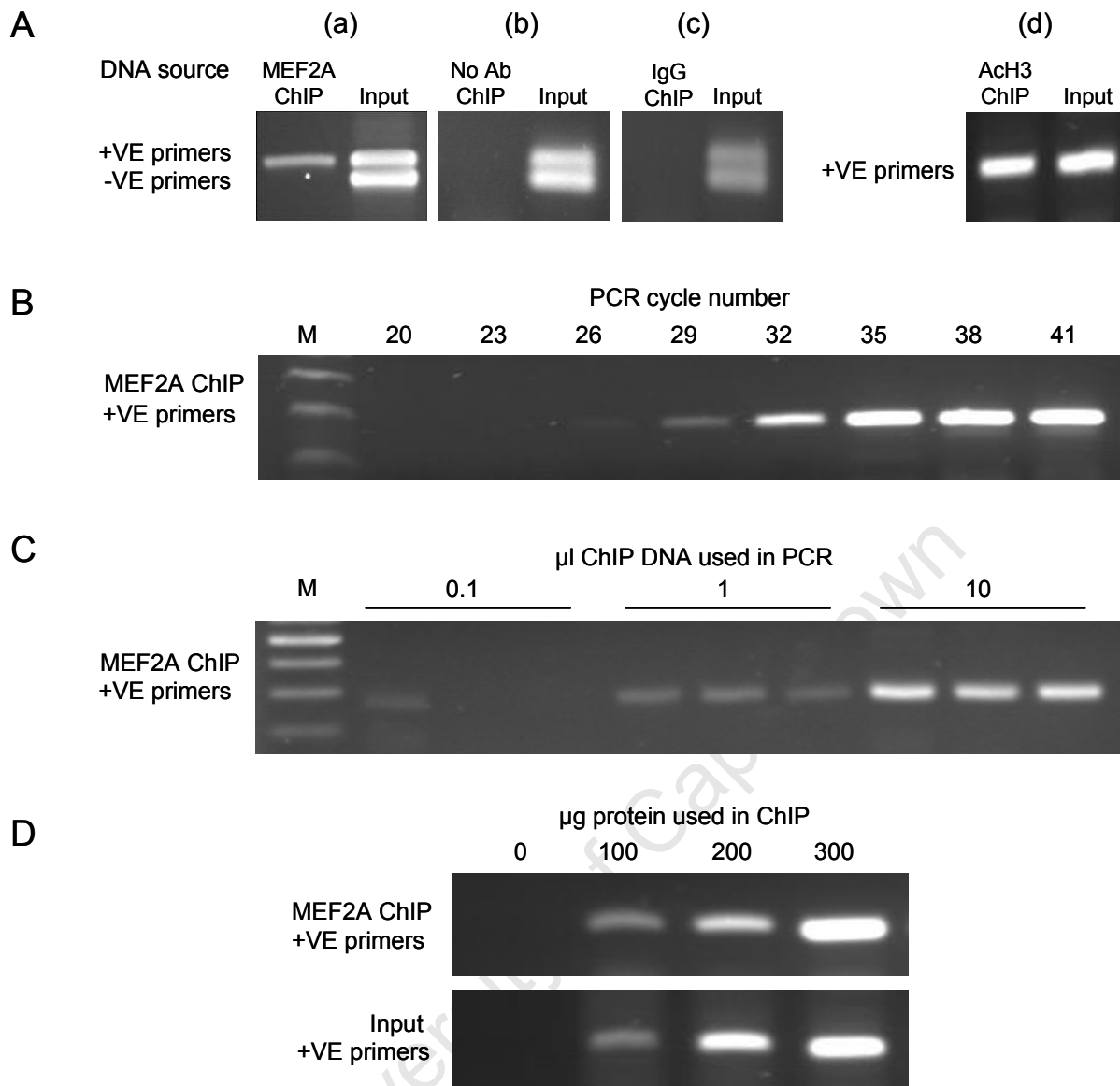


Figure 3.5. ChIP assay optimisation and negative controls. A. ChIP assays were performed on crosslinked rat muscle samples using an anti-MEF2A (a), anti-Acetyl Histone H3 (AcH3 - d), anti-mouse immunoglobulin (IgG - c) antibody or without an antibody (no Ab - b) and PCR was performed on co-immunoprecipitated DNA using primers which span the MEF2 binding domain in the *Glut4* promoter (+ve primers) and/or primers which span a region 5 kb downstream in the *Glut4* gene that does not contain a MEF2 *cis* element (-ve primers). Increasing the PCR cycle number (B) or increasing ChIP DNA template in the PCR (C) from MEF2A ChIP assays resulted in more abundant PCR product. D. The indicated amounts of protein sample were used in MEF2A ChIP assays. Input = DNA purified from a 1% volume of the input sample that was used for ChIP assays, prior to immunoprecipitation.

3.11 Polymerase Chain reaction

One μl of purified DNA was amplified by PCR using 0.5 U of SuperTherm Taq DNA polymerase (Medox Biotech) in 20 μl reactions containing 1 x SuperTherm reaction buffer; 0.2 mM dNTPs; 1-4 mM MgCl_2 ; and 0.2-0.5 μM forward and reverse primers. The reactions were thermally cycled in an XP cycler PCR machine (Bioer) using the following conditions: 1 cycle at 94°C for 10 minutes, followed by 30 - 35 cycles at 94°C for 30 seconds, 58-62°C (annealing temperature) for 30 seconds, and 72°C for 45 seconds followed by 1 cycle at 72°C for 5 minutes. The MgCl_2 and primer concentrations and the cycle number and annealing temperatures were optimised for each PCR.

3.12 Measurement of CaMK II activity

CaMK II activity was determined by measuring the incorporation of phosphate radioisotope (^{32}P) from [γ - ^{32}P] ATP into the specific CaMK substrate, Autocamtide 2, using a kit from Upstate Cell Signalling Solutions. Twenty-five μg of protein from lysates described in section 3.5, was incubated for 3 minutes at 30°C in a pre-heated reaction mix containing 4 mM 3-(N-morpholino) propanesulfonic acid (MOPS, pH 7.2); 5 mM β -glycerol phosphate; 1 mM Na_3VO_4 ; 1 mM DTT; 100 μM Autocamtide 2; 8 $\mu\text{g}/\text{ml}$ calmodulin; 2 μM PKA inhibitor peptide; 2 μM PKC inhibitor peptide; and 100 μM ATP (including 10 μCi of [γ - ^{32}P] ATP $\sim 3000\text{Ci}/\text{mmol}$) in a 50 μl reaction. Either 5 mM EGTA or 1.2 mM calcium chloride (CaCl_2) was included in the reaction mix to measure autonomous or *in vitro* maximal CaMK II activity, respectively. Ten μl of the reaction mix was spotted onto p81 phosphocellulose paper and the paper was air-dried and washed 3 x 10 minutes in 0.75% phosphoric acid. The counts per minute (CPM) of incorporated ^{32}P was measured using a Beckman scintillation counter (A). Background counts were determined from reactions without homogenate (B) or Autocamtide 2.

CaMK II activity was calculated as follows:

$$\frac{(A-B) \times \text{volume ratio}}{\text{SR} \times \text{time} \times \text{amount protein}} = \text{pmol } ^{32}\text{P incorporated/minute}/\mu\text{g protein}$$

In this case, A and B are defined above (CPM)

$$\text{volume ratio} = 50 \mu\text{l reaction}/10 \mu\text{l spotted onto p81 paper} = 5,$$

time = 3 minutes,

amount protein = 25 μ g

SR = specific radioactivity of the [γ - 32 P] ATP mixture.

= CPM /amount (pmoles) of 10 μ l [γ - 32 P] ATP mixture

In this case, CPM = CPM of 1 μ l [γ - 32 P] ATP mixture x 10,

amount = 5 x 10³ pmol

3.13 Immunohistochemical Analysis

CaMK II phosphorylation at Thr 286 was analysed in rat triceps muscle using immunohistochemistry. Twenty μ m sections of frozen muscle were cut using a cryostat (Leica Universal Microtome Cryostat HM 500) at -20°C , mounted onto 3-aminopropyltriethoxysilan coated glass slides, air-dried at -20°C and fixed in -20°C (3:1) methanol: glacial acetic acid for 10 minutes. Following four washes (each 5 minutes) in TBST, the sections were blocked in TBST containing 1% BSA, and incubated with an anti-pCaMK II (Thr 286) antibody overnight at 4°C . After washing 4 times for 5 minutes in TBST, slides were incubated in fluorochrome donkey anti-rabbit Cy3 (Dianova) for 2 hours at room temperature, washed 4 times for 5 minutes in TBST, layered with Mowiol containing the anti-fading *n*-propylgallate and covered with a glass coverslip. After drying for 20 minutes at room temperature, images were viewed under a confocal laser scanning microscope (LSM 510; Zeiss) equipped with a Plan-Apochromat 100 X oil immersion lens. Images were acquired with LSM 510 software and processed further with Photoshop (Adobe Systems).

3.14 Statistical analysis

Data from CHIP assays, Western blots, CaMK II activity assays and real time PCR are presented as means \pm SD. Statistical differences between treatments were determined using a one-way ANOVA or a Student's *t* test as appropriate. Significance was accepted at $P < 0.05$. When ANOVA showed a significant difference, post hoc analysis was performed using Fisher's least significant differences test. STATISTICA 7 software was used for these analyses.

CHAPTER FOUR

Exercise increases the binding of MEF2A to the *Glut4* promoter in rat skeletal muscle *in vivo*.

4.1 Introduction

As discussed in section 1.5, GLUT4 expression in skeletal muscle is regulated by MEF2 transcription factors, which have a conserved binding domain in the human, mouse and rat *Glut4* promoters (110; 110; 151; 151; 194; 202). McGee *et al.* (122) used EMSA to show that the binding of MEF2 factors to their *cis*-element in the *Glut4* promoter was increased in skeletal muscle immediately after human subjects completed a 60 minute bout of cycling. In those experiments, muscle nuclear protein extracts were incubated *in vitro* with a radio-labelled 18 bp oligonucleotide containing the MEF2 binding site in the human *Glut4* promoter and were separated by electrophoresis on an SDS-polyacrylamide gel. Two DNA-protein complexes were observed, both of which migrated slower when the nuclear extracts were pre-incubated with an anti-MEF2D antibody, indicating that MEF2D was present in both complexes. Because it had previously been shown using EMSA that MEF2 factors bind to the human and rat *Glut4* promoter as a MEF2A/MEF2D heterodimer or a MEF2D homodimer (110; 133; 194), the authors concluded that the complex with lower mobility comprised the MEF2A/MEF2D heterodimer, although they did not directly show that MEF2A was present in the complex. Furthermore they reported that exercise increased nuclear enrichment of MEF2A and increased the binding of MEF2A/MEF2D to the *Glut4* promoter (122). These observations were presented as evidence that MEF2-DNA binding might play an important role in exercise-induced GLUT4 expression (122).

It is however uncertain whether the increased binding of MEF2 factors to DNA that was observed in the EMSA experiments is a true reflection of the situation *in vivo*. Although EMSA is suitable for determining the extent to which a transcription factor interacts with DNA under *in vitro* conditions, and is particularly sensitive to changes in binding caused by alterations in the levels of the transcription factor, the *in vivo* binding conditions may not be adequately recreated. For example, binding of proteins which require DNA secondary structure, such as looping, to bring two distal binding sites into close proximity or those that require multi-protein complex formation to stabilize protein-DNA interactions, may not be recreated and detected in a gel

mobility shift analysis (210). Indeed, there is evidence to suggest that the interaction between MEF2 factors and GEF, which has a binding domain upstream from the MEF2 site in the human *Glut4* promoter, is essential for optimal *Glut4* promoter activity (96; 186). Furthermore, unlike in EMSA, where binding domains on DNA are freely accessible to transcription factors, *in vivo* DNA is bound to histone proteins through electrostatic forces and this interaction often limits transcription factor access to their binding sites (9; 55). Because EMSA detects whether proteins bind to short oligonucleotides that lack secondary structure, these experiments would be insensitive to any changes in chromatin structure that might restrict binding activity *in vivo*. Therefore an increase in nuclear translocation of MEF2A after exercise would manifest as increased binding in EMSA that would not necessarily bind *in vivo* because of the restrictions that may be imposed by chromatin structure. In light of the marked differences in environmental conditions that exist when transcription factors bind to DNA *in vitro* and when they bind *in vivo*, and to confirm that MEF2A is part of the complex that binds to the *Glut4* promoter after exercise, this study evaluated the binding of MEF2A to its *cis*-element on the rat *Glut4* promoter using *in vivo* ChIP assays on triceps muscles from rats that were exercised.

4.2 Methods

4.2.1 Animal care and exercise protocol. Wistar rats were familiarised to swimming, fasted overnight and swum for 5x17 minutes with a load of 4% body weight attached to their tails as described in section 3.2.2. At 0, 2, 6 or 18 hours after exercise, rats were anaesthetised and triceps muscles dissected out, immediately frozen in liquid nitrogen and stored at -80°C. Triceps muscle from rats that were not exercised were also harvested and used as controls.

4.2.2 Chromatin immunoprecipitation assays. ChIP assays were performed on the harvested muscle as described in section 3.9.2. Briefly, frozen muscles were ground in liquid nitrogen, cross-linked in 1% formaldehyde and the chromatin was sonicated to fragments less than 1000 bp in size. Chromatin that was cross-linked to MEF2A was co-immunoprecipitated with an anti-MEF2A antibody, de-crosslinked, and the DNA purified and PCR amplified using primers spanning the MEF2 binding site in the rat *Glut4* promoter. 1% of the sample used for each ChIP assay was purified and PCR amplified. Negative control experiments that omitted the antibody or used a non specific antibody (anti-IgG) were performed in parallel and PCR was also performed on DNA from MEF2A ChIP assays using primers which span a region approximately 5 kb downstream from the *Glut4* start site that does not contain a MEF2 *cis*-element.

4.2.3 Western Blotting. The contents of MEF2A, GLUT4 and α -tubulin in harvested muscle samples were assayed by western blot as described previously in sections 3.5 (muscle homogenisation) and 3.6.1 (western blots). Briefly, frozen muscle samples were homogenised in HES buffer and the proteins separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes using electrophoresis. The membranes were incubated with primary antibodies (anti-MEF2A and anti-GLUT4), washed and incubated with appropriate HRP-conjugated secondary antibodies according to Table 3.3. The protein/antibody complexes were incubated with a substrate of HRP (ECL reagent) and the signals captured using radiographic film and quantified using UN-SCAN IT gel software. To control for possible differences in the amounts of total protein that were loaded onto the SDS polyacrylamide gel, the membranes were stripped of antibodies (section 3.6.2) and re-probed for α -tubulin (a protein that is unaffected by the treatments used in the experiments). Signal intensities for GLUT4 and MEF2A were normalised to α -tubulin and expressed relative to a control in each experiment.

4.2.4 Real Time quantitative PCR. Relative GLUT4 mRNA levels were measured from rat muscles using real time quantitative PCR as described in section 3.8. Briefly, total RNA was isolated from frozen muscles using Tri reagent (Ambion) as described in section 3.8.1. RNA concentration was determined by measuring the absorbance at 260nm and the integrity of the RNA was confirmed by separating RNA on a 1% formaldehyde-agarose gel stained with ethidium bromide and observing intact ribosomal RNA (section 3.8.2). RNA was cleared of genomic DNA contamination by incubating with DNase 1 (Promega) and cDNA was generated from 1 μ g of total RNA using MMLV reverse transcriptase (Promega) as described in section 3.8.3. Real time PCR was performed using primers which amplify a region in the GLUT4, GAPDH or RS12 gene. Details of the PCR protocol are found in section 3.8.4 and the primer sequences are listed in Table 3.4. Relative GLUT4 mRNA levels were calculated based on the threshold cycle of the PCR and were normalized to RS12 and GAPDH house keeping genes and expressed relative to a control in each experiment as described by Livak *et al.* (112).

4.2.5 Glycogen assays: Glycogen content was determined as described in section 3.7. Briefly, glycogen from muscle was extracted in 10% KOH, precipitated in 80% ethanol and hydrolysed to glucose equivalents in 2 N HCl. The glucose concentration of the sample was measured using a glucose analyser (Beckman) and glycogen concentration was calculated using the formula described in section 3.7.

5.2.6 Statistic analysis: Data are presented as means \pm SD. Statistical differences between treatments were determined using a one-way ANOVA and differences between groups were confirmed with a Fisher's Least Significant Differences post hoc test as described in section 3.14. Significance was accepted at $P < 0.05$.

4.3 Results

4.3.1 Glycogen levels are decreased and GLUT4 mRNA and protein content is increased in rat triceps muscle after exercise. Rats were exercised by a bout of moderate intensity swimming and triceps muscles were dissected out at 0, 2, 6 or 18 hours after exercise and analysed for glycogen and GLUT4 mRNA and protein. Figure 4.1A shows that glycogen was significantly reduced by 70% immediately after the swimming compared to sedentary controls ($P = 0.001$). Muscle glycogen is one of the major fuels that is mobilised to support physical activity during exercise of moderate to high intensity (65). The decreased muscle glycogen content therefore indicates that the exercise was of sufficient intensity to mobilise glycogen stores in triceps muscles. GLUT4 mRNA was significantly increased by ~2-fold at 6 hours post-exercise ($P = 0.008$) but was unchanged at 0 or 2 hours after exercise (Figure 4.1B). It is however possible that an increase in GLUT4 mRNA occurred sooner than 6 hours after exercise. GLUT4 protein content was significantly elevated by ~1.7-fold at 18 hours after exercise ($P = 0.005$) but was unchanged at 0 or 6 hours post-exercise (Figure 4.1C). Collectively, these results demonstrate that the exercise protocol was of sufficient intensity to activate the signalling pathways that increase GLUT4 expression.

*4.3.2 A bout of exercise increases MEF2A binding to its cis-element on the rat *Glut4* promoter in vivo but does not change total MEF2A content.* ChIP assays, using an antibody directed against MEF2A and primers that amplify the MEF2 binding site in the *Glut4* promoter, were performed on triceps muscles from exercised rats. The amount of MEF2A that was bound to this site *in vivo* tended to increase at 0 hours after exercise ($P = 0.19$), was significantly elevated at 6 hours post exercise ($P = 0.003$) but was not different at 18 hours after exercise, compared to sedentary control rats (Figure 4.2A, a). The reason that MEF2A binding was not significantly increased immediately after exercise may be attributed to the high variability of the data which was acquired using semi quantitative conventional PCR. Unfortunately quantitative Real Time PCR was not available at the time of the experiments. As discussed in section 3.9.3, when an anti-IgG antibody was used, or the antibody was omitted from the assay, no detectable signal was

produced (Figure 3.5A), which verifies that the conditions used for the ChIP assay are specific to the anti-MEF2A antibody. Furthermore the assay produced no detectable signal when the MEF2A antibody was used with primers that amplify a region in the *Glut4* gene which is 5 kb from the MEF2 binding site and does not contain a MEF2 *cis* element (Figure. 4.2A, b). This ensures that non-specific chromatin does not co-immunoprecipitate with the anti-MEF2A antibody and that the chromatin fragments that were co-immunoprecipitated with MEF2A did not extend further than 5 kb from the *Glut4*-promoter MEF2 binding site. Total MEF2A content in the muscles was not different at 6 hours after exercise compared to controls (Figure 4.2B, $P = 0.786$).

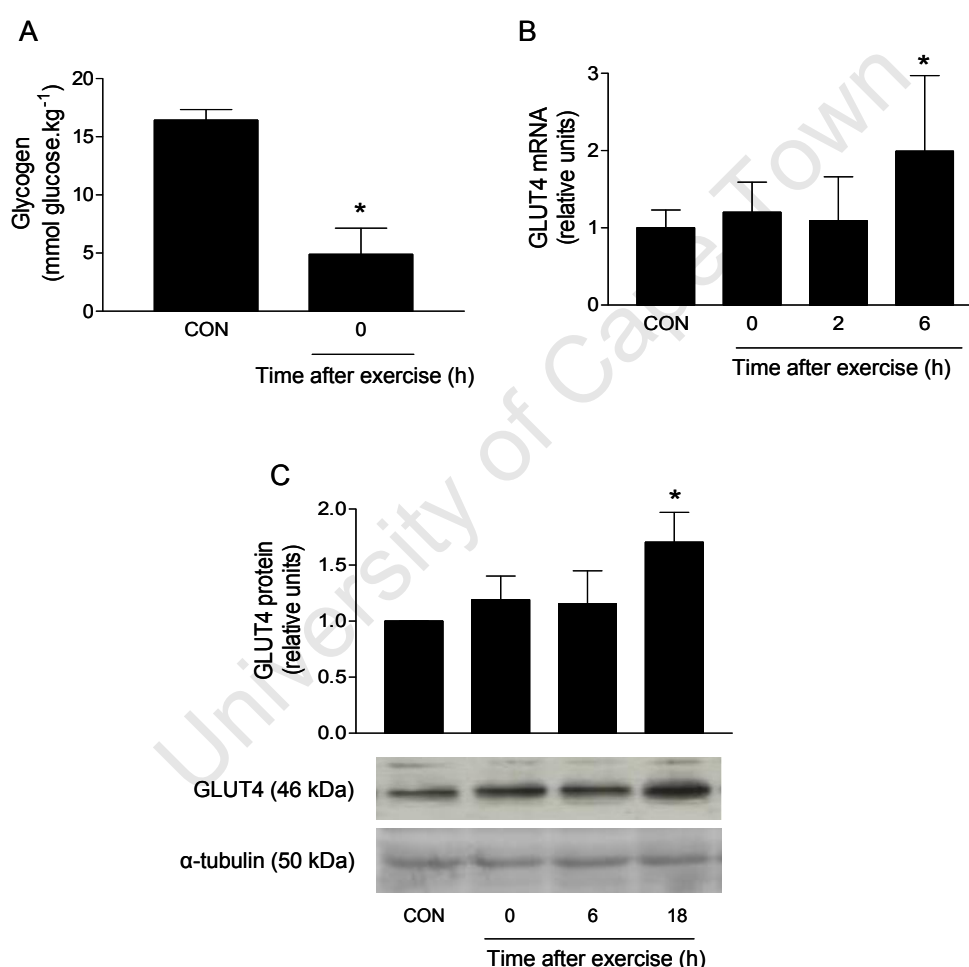


Figure 4.1. A bout of exercise decreases glycogen and increases GLUT4 mRNA and protein contents in skeletal muscle. Triceps muscles were isolated from rats at 0, 2, 6 or 18 hours after completing a 5 x 17 minute swim or from rats that did not swim (CON). A. Muscle glycogen content was determined as described in section 4.2.5. $n = 3$, $* P = 0.001$ vs. CON. B. GLUT4 mRNA was measured by qRT-PCR and normalised to GAPDH mRNA (section 4.2.4). $n = 5$, $* P = 0.008$ vs. CON. C. GLUT4 and α -tubulin protein content was measured by Western blot as described in section 4.2.3. Shown are representative GLUT4 and α -tubulin blots. Signals for GLUT4 in the histogram were normalised to signals for α -tubulin. $n = 4$, $* P = 0.005$ vs. CON. Data are presented as means \pm SD.

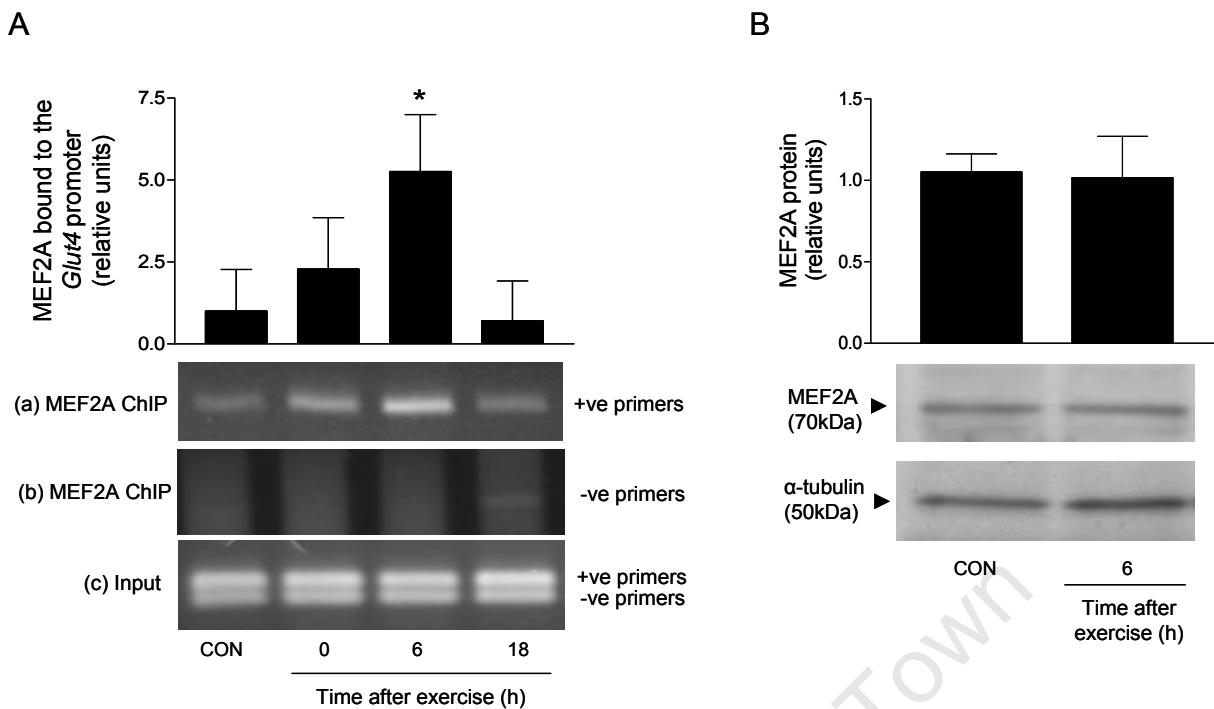


Figure 4.2. Exercise increases the binding of MEF2A to the *Glut4* promoter but does not change MEF2A protein content. Triceps muscles were isolated at 0, 6 or 18 hours after rats completed a 5 x 17 minute swim or from control rats that did not swim (CON). A. ChIP assays using an anti-MEF2A antibody were performed on crosslinked muscles and co-immunoprecipitated DNA was amplified by PCR using primers which span the MEF2 site in the *Glut4* promoter (+ve primers) (a) as described in section 4.2.2. The histogram presents data from 4 independent experiments, * $P = 0.003$ vs. CON. PCR using primers that amplify a region in the *Glut4* gene that does not contain a MEF2 *cis*-element (-ve primers) was performed on MEF2A chip samples (b). Chromatin from an aliquot of sample that did not undergo immunoprecipitation was PCR amplified using +ve and -ve primers and used for comparison (Input) (c). B. Representative western blots showing the contents of MEF2A and α -tubulin in rat muscles taken 6 hours after swimming or from controls (CON). The histogram presents signals for MEF2A protein normalised to α -tubulin from 6 independent experiments. Data are presented as means \pm SD.

4.4 Discussion

The major novel finding of the study in this chapter is that, *in vivo*, the amount of MEF2A bound to its domain in the *Glut4* promoter increases in rat skeletal muscle in a time-dependent manner following a bout of exercise (Figure 4.2A). The increase in MEF2A binding was unlikely to be due to an increase in MEF2A expression because total MEF2A content, as assayed by western blot, was not elevated at 6 hours post exercise (Figure 4.2B). A study by Holmes *et al.* (71) showed that increased binding of MEF2A to its consensus sequence on the *Glut4* gene can occur due to increased translocation of MEF2 to the nucleus with only a modest increase in total

MEF2A content. In fact, McGee *et al.* (122) demonstrated that an acute bout of exercise increases nuclear abundance of MEF2A in human skeletal muscle without changing total MEF2A. The above observations led to the hypothesis that exercise might increase the binding of MEF2A to the *Glut4* promoter by increasing its nuclear translocation. As changes in nuclear MEF2A content were not measured in this thesis, results presented in this chapter are unable to confirm or refute this hypothesis.

The increased DNA binding may also be due to increased access of the transcription factors to their binding domain in the *Glut4* promoter. *In vivo*, access of transcription factors to their binding domains on chromatin is often dependent on post-translational modifications of histones within the nucleosomes that surround the gene (9; 102). For example, hyper-acetylation of the lysine residues on the N terminal tails of histone H3 or H4 neutralises the positive charge on these residues and weakens the interaction between the histones and the negatively charged phosphate backbone of the DNA (56; 58; 144; 146). This modification facilitates the displacement of histones by transcription factors and results in increased transcription (58). It is possible that the increased MEF2A binding that was observed in this study was also facilitated by increased accessibility of the transcription factor to chromatin, perhaps due to hyper-acetylation of histones within the *Glut4* gene.

Using an antibody directed against MEF2D in EMSA, McGee *et al.* (122) provided indirect evidence that the MEF2A/MEF2D heterodimer binds to its *cis*-element on the human *Glut4* gene and that this binding increased after exercise. The data from this chapter, which was obtained using a MEF2A antibody in ChIP assays, supports their conclusion that MEF2A is present in the complex that binds to the *Glut4* gene in response to exercise. However, in their experiments the increase in binding was detectable immediately after exercise. In the present study a small but non-significant increase at this time point was seen (Figure 4.2A). Instead, a significant increase in MEF2A binding was observed at 6 hours post exercise. It is possible that the increased binding may have started sooner than 6 hours after exercise but at the time of the experiments it was not feasible to perform ChIP assays on muscles collected at these earlier time points.

The discrepancy in MEF2A binding at 0 hours post exercise between McGee *et al.* (122) and the present study could have been due to differences in species (human vs. rat), muscle (vastus lateralis vs. triceps) and/or the assays used to measure DNA binding (EMSA vs. ChIP). EMSA detected the extent to which MEF2 factors in nuclear homogenates interact with an

oligonucleotide that contains the MEF2 *cis*-element *in vitro* and would therefore be insensitive to changes in chromatin structure that could restrict access of transcription factors to their binding domains *in vivo*. Numerous other studies have indicated that the binding of MEF2 factors to DNA *in vitro* is highly correlated with nuclear MEF2A content (133; 194). Therefore the increased nuclear translocation of MEF2A that was observed by McGee *et al.* (122) would immediately be detected as increased binding to the oligonucleotide by EMSA but there may be a lag *in vivo* because of the additional time required to permit chromatin remodelling.

In summary this chapter presented the novel finding that the increase in GLUT4 mRNA and protein expression observed in rats after exercise is preceded by an increase in the binding of MEF2A to its *cis*-element in the *Glut4* promoter *in vivo*. Because numerous other reports also indicate that increased binding of MEF2 to gene promoters is associated with increased expression of the gene (122; 181), MEF2A binding to the *Glut4* promoter may play an important regulatory role in GLUT4 expression during exercise.

CHAPTER FIVE

CaMK is necessary for exercise-induced binding of MEF2A to its binding site in the *Glut4* promoter

5.1 Introduction

The findings in chapter 4 suggest that increased binding of MEF2A to the *Glut4* promoter might play an important regulatory step for GLUT4 expression during exercise. The signals that regulate MEF2A binding to the *Glut4* promoter and GLUT4 expression in response to exercise have however, not been completely elucidated. Previous work in cell culture has indicated that CaMK might be involved. For example, Ojuka *et al.* (150) incubated L6 myotubes (a rat muscle cell line) with caffeine to raise intercellular Ca^{2+} levels to a similar extent that is observed during muscle contraction and noticed an increase in GLUT4 protein content. This increase was completely blocked when the CaMK inhibitor, KN93, was included in the medium. Scicchitano *et al.* (181) also showed that CaMK is necessary for MEF2A binding to its binding site in the myogenin and MCK promoters during ARG-vasopressin induced differentiation of L6 myotubes. Because CaMK II activity is elevated during exercise (173; 174), it seems reasonable to speculate that CaMK II might also regulate the binding of MEF2A to the *Glut4* promoter and increase GLUT4 expression after exercise. The first purpose of the study presented in this chapter therefore, was to determine whether CaMK was necessary for exercise to increase MEF2A binding to the *Glut4* promoter and to enhance GLUT4 expression. To investigate this, rats were injected with KN93 to inhibit CaMK activity in skeletal muscle prior to completing an intermittent swimming session. CaMK inhibition completely blocked the increased binding of MEF2A to the *Glut4* promoter and reduced the increases in GLUT4 mRNA and protein content in response to exercise.

The mechanism by which CaMK increases GLUT4 expression and binding of MEF2A to DNA is also elusive. However there is evidence showing that CaMK might cause the acetylation of the histones within nucleosomes that surround MEF2 binding domains in gene promoters (181). As mentioned previously in section 1.6, acetylation of histones within nucleosomes relaxes chromatin structure making it easier for RNA polymerases and transcription factors to access their binding domains on the promoter regions of target genes (56; 58; 144; 146). A strong correlation

between gene expression, acetylation of histone H4 at MEF2 sites and increased binding of MEF2 to its *cis*-element has been elegantly demonstrated by Scicchitano *et al.* (181) using myogenin and MCK genes during Arg-Vasopressin (AVP)-induced differentiation of L6 myoblasts. Furthermore, treatment of these differentiating L6 myoblasts with KN62, an inhibitor of CaMK, reduced the AVP-induced increases in gene expression, MEF2 binding and histone H4 acetylation at MEF2 sites on both genes. These findings provide strong evidence that CaMK activation is essential for both acetylation of histone proteins at MEF2 sites and increased binding of MEF2 to its binding domain on DNA. The effect of exercise or CaMK activity on the acetylation pattern of histones within various segments of the *Glut4* gene has not been characterized to date. Hence the second purpose of the study presented in this chapter was to determine whether exercise increases the acetylation of histones in the *Glut4* promoter in rat skeletal muscle. A marked hyperacetylation of histones near the MEF2 binding site in the *Glut4* promoter after a bout of swimming was observed. This observation provided the basis for the third purpose of the study, which was to determine whether the exercise-induced increase in histone acetylation required CaMK activity.

5.2 Materials and methods

5.2.1 Animal care and exercise protocol. Full details of the protocol used to swim rats are found in section 3.3.2. Briefly, rats were familiarised to swimming, rested for 6 days and fasted overnight. The following day, rats were used in 1 of 2 experiments: In the first experiment to determine whether CaMK II activity was increased after exercise, rats completed 5 x 17 minutes of swimming carrying a load of 4% body weight. Triceps muscles were dissected out from anaesthetised rats at 0, 0.5 or 2 hours after exercise. Rats that did not perform the final exercise session were used as controls. A second experiment was designed to test whether CaMK activity was necessary for the exercise-induced adaptations in GLUT4 expression. Rats in this experiment were randomly divided into three groups: The first and second groups were injected (i.p.) with 5 mg.kg⁻¹ KN93 to inhibit CaMK II activity or an equivalent volume of saline, respectively, 30 minutes prior to exercise. In experiments to assess GLUT4 protein expression after exercise, the exercise protocol was performed on two consecutive days to ensure a more robust increase in protein levels. The third group of rats was injected with saline but did not participate in the final swimming session (controls). Rats were anaesthetised and triceps muscles were dissected out at 0 or 6 hours after exercise on the first day or 18 hours after exercise on the second day.

5.2.2 Chromatin immunoprecipitation assays. ChIP assays were performed on rat muscles to measure MEF2A binding to its site in the *Glut4* promoter and the acetylation of Histone H3 in the *Glut4* promoter as described in section 3.9.2. Briefly, frozen muscles were ground in liquid nitrogen, cross-linked in 1% formaldehyde and the chromatin was sonicated to fragments less than 1000 bp in size. Samples were immunoprecipitated with an anti-MEF2A or an anti-Acetyl Histone H3 antibody and PCR was performed on the co-immunoprecipitated chromatin using primers spanning the MEF2 binding site in the rat *Glut4* promoter. 1% of the sample used for each ChIP assay was also purified and PCR amplified. Negative control experiments that omitted the antibody or used a non specific antibody (anti-IgG) were performed in parallel and PCR was also performed on DNA from MEF2A ChIP assays using primers which span a region approximately 5 kb downstream from the *Glut4* start site that does not contain a MEF2 *cis*-element.

5.2.3 Western Blotting. MEF2A and GLUT4 protein contents were assayed by western blot as described in section 4.2.3 and were normalised to values from α -tubulin blots.

5.2.4 Real Time quantitative PCR. Relative GLUT4 mRNA was measured using real time quantitative PCR and normalised to mRNA values from GAPDH (presented in this chapter) and RS12 (data not shown) as described in section 4.2.4.

5.2.5 CaMK II activity. CaMK II activity assays were performed by measuring the incorporation of ^{32}P into the CaMK II substrate, Autocamtide 2, as described in section 3.12. Briefly, muscle homogenates were generated as described in section 3.5 and were incubated with Autocamtide 2; calmodulin; $[\gamma\text{-}^{32}\text{P}]$ ATP and either CaCl_2 (for maximal *in vitro* CaMK activity) or EGTA (for autonomous CaMK activity). ^{32}P that was incorporated into the substrate was measured and CaMK II activity was calculated according to the formula in section 3.12.

5.2.6 Immunohistochemistry. CaMK II phosphorylation at Thr 286 was analysed in rat triceps muscle using immunohistochemistry as described in section 3.13. Briefly, 20 μm sections of frozen muscle were cut using a cryostat and mounted onto glass slides. The sections were blocked in TBST containing 1% bovine serum albumin and incubated with an anti-pCaMK II (Thr 286) antibody overnight at 4°C. After washing in TBST, slides were incubated in fluorochrome donkey anti-rabbit Cy3, re-washed and layered with Mowiol containing the anti-fading *n*-propylgallate and covered with a glass coverslip. Images were viewed under a confocal laser scanning microscope equipped with a 100X oil immersion lens.

5.2.6 *Statistical analysis:* Data are presented as means \pm SD. Statistical differences between treatments were determined using a one-way ANOVA and differences between groups were confirmed with a Fisher's Least Significant Differences post hoc test as described in section 3.14. Significance was accepted at $P < 0.05$.

5.3 Results

5.3.1 *CaMK II activity increases transiently after exercise.* To begin to investigate whether CaMK might play a role in the exercise-induced binding of MEF2A to the *Glut4* promoter, CaMK II activity was measured in rat triceps muscle at various time points after a bout of exercise. Autonomous CaMK II activity was significantly elevated \sim 1.7-fold immediately after exercise compared to sedentary controls ($P = 0.44$) but declined rapidly thereafter (Figure 5.1A). Maximal *in vitro* CaMK II activity did not change significantly during the 2 hours post-exercise (Figure 5.1B). As discussed in section 1.8 autonomous CaMK II activity is associated with the phosphorylation of CaMK II at Thr 286 (174). Western blots and immunohistochemical staining of muscle sections using an antibody that recognises phosphorylated CaMK II at Thr 286, indicates that there is an increase in CaMK II phosphorylation, immediately after exercise compared to controls (Figure 5.1E and F).

KN93 is a compound which has been used to inhibit CaMK II activity in cultured muscle cells, isolated muscle preparations and live animals (10; 137; 150; 221). Administration of KN93 to rats, 30 minutes prior to beginning the swimming session completely blocked the increase in autonomous CaMK II activity that was observed in triceps muscles after exercise (Figure 5.1C), but did not influence *in vitro* maximum CaMK II activity (Figure 5.1D). Furthermore, phosphorylation of CaMK II at Thr 286 was prevented by KN93 (Figure 5.1E and F). The drug had no visible side effects on rats and did not appear to impair exercise performance. Collectively these results indicate that the dose of KN93 used, effectively blocked CaMK II activity and phosphorylation in triceps muscle after exercise.

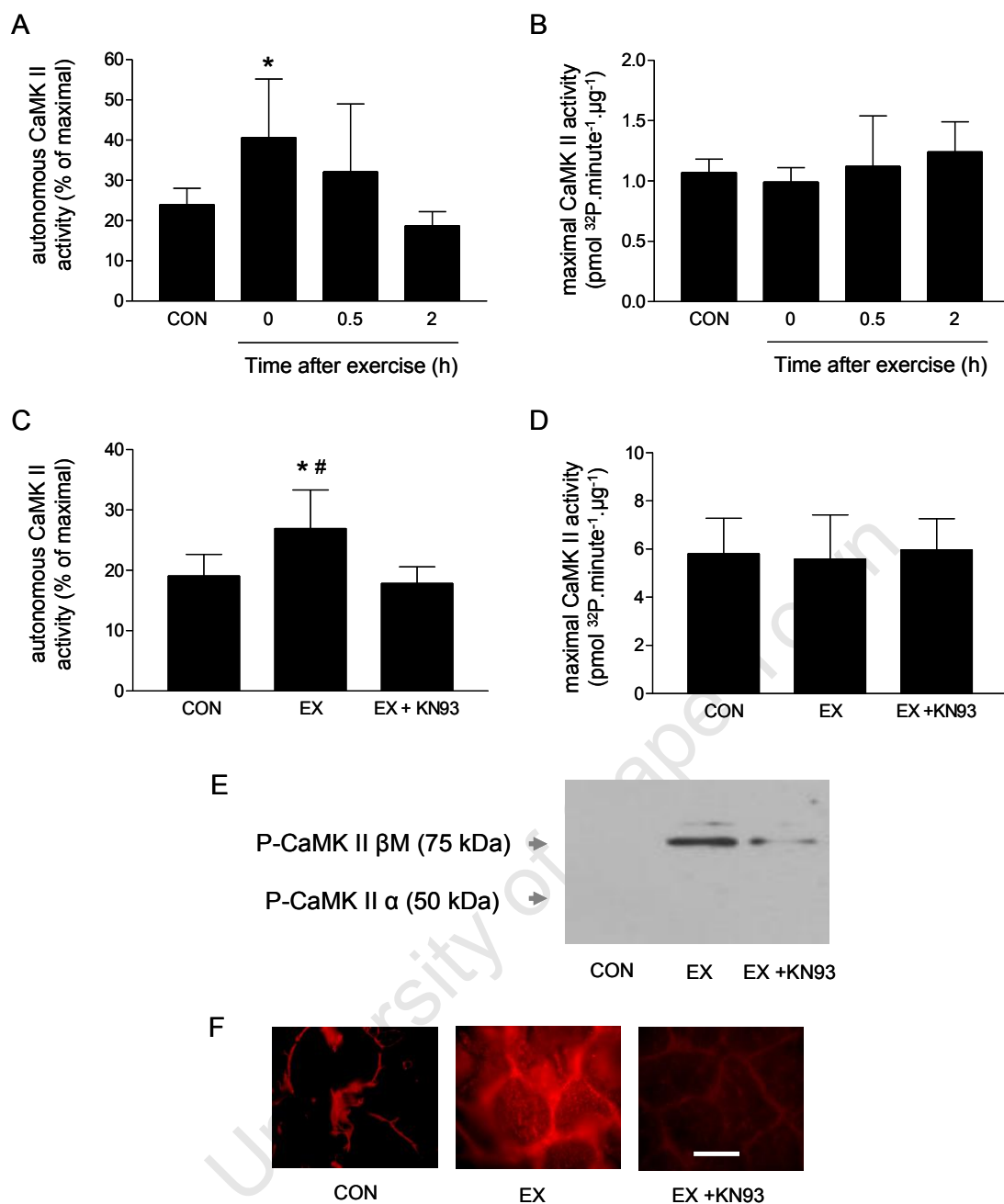


Figure 5.1. CaMK II activity and phosphorylation are increased after exercise. Autonomous (Ca^{2+} independent) and *in vitro* maximal (Ca^{2+} dependent) CaMK II activity was measured in rat triceps muscles as described in section 3.7 and expressed as a percentage of maximal CaMK II activity (A + C) or presented as absolute maximum values (B + D). A + B: Rats were swum for 5 x 17 minutes and muscles were isolated at 0, 0.5 or 2 hours after the final bout. CON rats were not exercised. $n = 4$ independent experiments, * $P = 0.044$ vs. CON. C+D. Rats were injected with KN93 (EX+KN93) or saline (EX), 30 minutes prior to the swimming session. CON rats were injected with saline but were not exercised. Muscles were isolated immediately after rats completed the swim. $n = 4$ independent experiments, * $P = 0.04$ vs. CON; # $P = 0.02$ vs. EX+KN93. Data are presented as means \pm SD. E. Representative western blot of triceps muscle from rats in C and D using a phosphorylated CaMK II Thr 286 antibody. F. Representative immunohistochemical stains of triceps muscle from rats in C and D showing phosphorylated CaMK II at Thr 286. Scale bar = 10 μM .

5.3.2 The exercise-induced increase in GLUT4 expression is reduced by CaMK inhibition.

Rats were made to swim for 5 X 17 minutes carrying a load of 4% body weight on one or two consecutive days having received KN93 or saline prior to each exercise session. GLUT4 mRNA was measured from triceps muscle 6 hours after exercise on the first day whereas GLUT4 protein was measured 18 hours after exercise on the second day. Exercise significantly increased GLUT4 mRNA by 2.2-fold compared to sedentary controls (Figure 5.2A). KN93 administration reduced the exercise-induced increase in GLUT4 mRNA to ~1.6 -fold relative to sedentary controls, however this reduction was not significantly different as a result of the high variability of the data ($P = 0.18$, exercised vs. exercised with KN93). It is possible that a significant reduction in mRNA would be observed with a larger sample size. Exercise increased GLUT4 protein levels to ~ 1.8 – fold compared to sedentary controls as expected (Figure 5.2B; $P = 0.008$). Administration of KN93 significantly reduced this increase to ~1.2 -fold above controls ($P = 0.03$, exercise vs. exercise with KN93). Collectively, these results demonstrate that CaMK activation is at least partly responsible for the increase in GLUT4 protein expression after exercise.

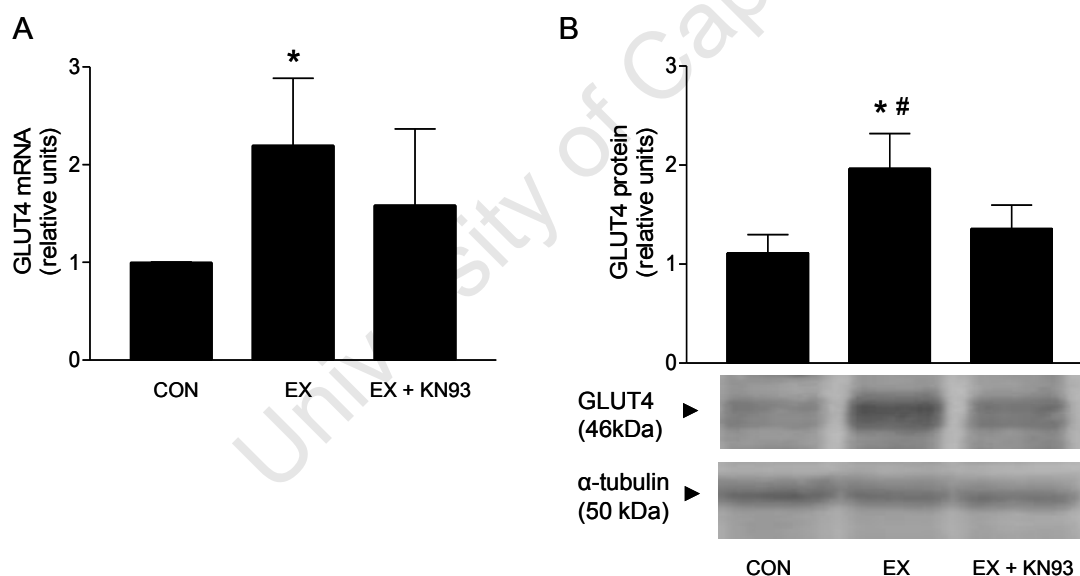


Figure 5.2. The increase in GLUT4 mRNA and GLUT4 protein after exercise is reduced by KN93. Rats completed a 5 x 17 minute swim session on one or two consecutive days. Rats were injected with KN93 (EX+KN93) or an equivalent volume of saline (EX) 30 minutes prior to each swim. CON rats were injected with saline but kept sedentary. A. GLUT4 mRNA was measured 6 hours after the first swim session by qRT-PCR (see section 4.2.4). The histogram presents values for GLUT4 normalised to GAPDH mRNA. $n = 5$ independent experiments, * $P = 0.02$ vs. CON. B. GLUT4 and α -tubulin protein was measured 18 hours after the second swim session by western blot (see section 4.2.3). Shown are representative GLUT4 and α -tubulin blots and a histogram presenting values for GLUT4 protein normalised to values for α -tubulin. $n = 6$ independent experiments, * $P = 0.008$ vs. CON; # $P = 0.03$ vs. EX+KN93. Data are presented as means \pm SD.

5.3.4 Inhibition of CaMK II activity blocks the exercise-induced increase in MEF2A binding to the *Glut4* promoter. ChIP assays were performed on muscles dissected from rats that were administered KN93 prior to exercise. DNA-bound MEF2A from muscle homogenates was co-immunoprecipitated with an anti-MEF2A antibody and the co-immunoprecipitated DNA was purified and amplified by PCR using primers spanning the MEF2 site on the *Glut4* promoter. There was ~1.9-fold more MEF2A bound to the *Glut4* promoter 6 hours after exercise, compared to rats that did not exercise (Figure 5.3a; $P = 0.002$). KN93 significantly reduced this binding to levels comparable to the sedentary rats ($P = 0.007$, exercise vs. exercise with KN93). When the assay was conducted with a non-specific antibody (anti-IgG) or without antibody (Figure 3.4A); or when primers that amplify a region 3 kb from the MEF2 site in the *Glut4* gene were used (Figure 5.3b), no PCR signals were obtained; demonstrating that the assays were sensitive to, and specific for, the MEF2 site in the *Glut4* promoter. These results demonstrate that the increase in MEF2A binding to the *Glut4* promoter after exercise, requires CaMK activation.

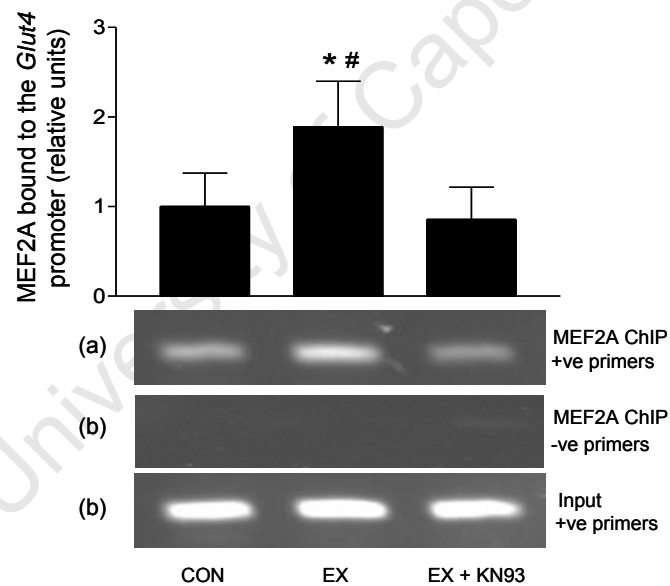


Figure 5.3. KN93 blocks the increase in MEF2A binding to the *Glut4* promoter after exercise. Rats were injected with KN93 (EX+KN93) or an equivalent volume of saline (EX) and completed a 5 x 17 minute swim. CON rats were injected with saline but kept sedentary. ChIP assays using a anti-MEF2A antibody were performed on muscles dissected from rats 6 hours after exercise and PCR was performed on co-immunoprecipitated DNA using primers which amplify the MEF2 site in the *Glut4* promoter (+ve primers) (a) as described in section 5.2.2. The histogram presents MEF2A ChIP data from 4 independent experiments, * $P = 0.02$ vs. CON; # $P = 0.007$ vs. EX+KN93. PCR using primers that amplify a region in the *Glut4* gene that does not contain a MEF2 *cis*-element (-ve primers) was performed on DNA from MEF2A ChIP assays (b). Chromatin from an aliquot of sample that did not undergo immunoprecipitation was PCR amplified and used for comparison (Input) (c).

5.3.5 *Exercise causes hyper-acetylation of histone H3 at the MEF2 binding site of the Glut4 gene and CaMK inhibition blocks this increase.* To further investigate the mechanism by which KN93 blocks the exercise-induced increase in MEF2A binding to the *Glut4* gene, the level of acetylation of histone H3 within the *Glut4* promoter was investigated immediately after exercise. ChIP assays using an anti-acetyl Histone H3 antibody and primers that amplify the region spanning the MEF2 binding site in the *Glut4* promoter were used on muscles from rats that were administered KN93 and exercised. Exercise significantly increased the acetylation of histone H3 within the *Glut4* promoter 1.75-fold compared to sedentary control rats (Figure 5.4; $P = 0.002$). Administration of KN93 prior to exercise significantly reduced this increased acetylation to ~1.25-fold above controls ($P = 0.01$, exercise vs. exercise with KN93). When the assay was conducted with a non specific antibody (anti-IgG) or without antibody (Figure 3.4A) negligible PCR products were obtained, which ensured that the assay was specific to the anti-acetylated histone H3 antibody. These results demonstrate that the increased acetylation of histone H3 within the *Glut4* promoter after exercise requires CaMK activity.

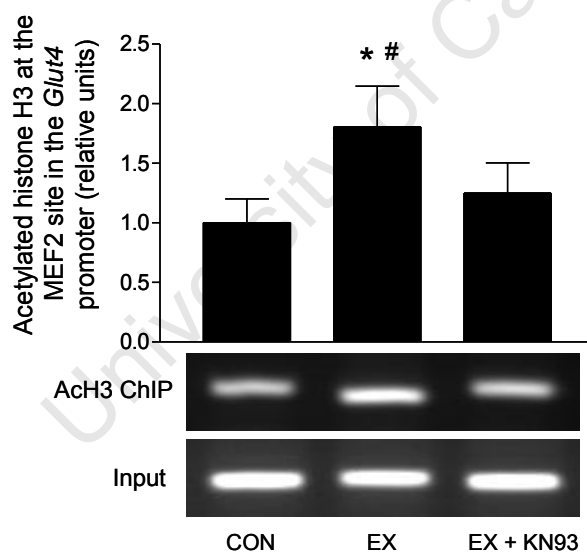


Figure 5.4. The increase in the acetylation of H3 histones in the *Glut4* promoter after exercise is attenuated by KN93. Rats were injected with KN93 (EX+KN93) or an equivalent volume of saline (EX) and completed a 5 x 17 minute swim. CON rats were injected with saline but kept sedentary. ChIP assays using an anti-Acetyl Histone H3 (ACh3) antibody were performed on muscles taken immediately after exercise and PCR was performed on the co-immunoprecipitated DNA using primers which amplify the MEF2 binding site in *Glut4* promoter. The histogram presents ACh3 ChIP assays from 4 independent experiments * $P = 0.002$ vs. CON; # $P = 0.01$ vs. EX+KN93. Chromatin from an aliquot of each sample that did not undergo immunoprecipitation was PCR amplified and used for comparison (Input).

5.4 Discussion

It is well established that a bout of exercise increases GLUT4 mRNA and protein content in rat and human skeletal muscle (164; 229) and, as reported in Chapter 4 of this thesis, these adaptations are preceded by increased binding of MEF2A to its binding site on the *Glut4* promoter in rats. This chapter used rats to demonstrate for the first time that inhibition of CaMK activity by KN93, prevented the increase in MEF2A binding to the *Glut4* promoter that is normally seen after exercise (Figure 5.3), and reduced the exercise-induced increase in GLUT4 mRNA and protein levels (Figure 5.2A and 5.2B). To explore the mechanisms whereby KN93 prevented this increased GLUT4 expression and MEF2A binding, the acetylation of histone H3 in the *Glut4* gene was investigated. It was demonstrated for the first time that exercise increased the acetylation of histone H3 within the *Glut4* promoter in skeletal muscle immediately after exercise and KN93 markedly reduced this acetylation (Figure 5.4). Taken together however, the data suggest that CaMK plays an important role in exercise-induced GLUT4 expression and does so by increasing MEF2A binding to the *Glut4* promoter and by causing the acetylation of histones within this site. A potential limitation of the experiments in this chapter is that the effects of KN93 on the basal levels of the measured variables was not evaluated making it difficult to interpret the true effects of KN93.

Wolffe and Hayes (219) describe two models by which hyper-acetylated lysine residues on histone H3 or H4 tails might affect transcriptional activity. Firstly, acetylation of these residues neutralizes the positive charge on the histone N termini, thus weakening histone-DNA contacts thereby reducing chromatin compaction (220). This loosening of chromatin structure facilitates access of transcription factors and RNA polymerases to their binding domains on DNA (105; 160). Secondly, some transcriptional co-activators possess bromodomains that interact specifically with acetylated lysines in histone H3 and H4 tails, therefore hyper-acetylated histones themselves may also recruit or anchor transcriptional co-activator complexes to genes leading to increased transcription (34; 100; 189; 203). Consistent with this theory, a ~ 2-fold increase in the acetylation of histone H3 in the neighbourhood of the MEF2 binding domain on the *Glut4* gene (Figure 5.4) and a corresponding ~ 2-fold increase in the amount of MEF2A that was bound to this domain (Figure 5.3) was observed in response to exercise.

The level of acetylation of histones in nucleosomes is a balance between the activity of histone acetyl transferases (HATs), which acetylate histones, and class II histone deacetylases (HDACs), which remove the acetyl groups (7; 124). Class II histone deacetylases such as HDAC4

and HDAC5 bind to and repress MEF2A transcriptional activity by deacetylating histones within the promoters of MEF2 regulated genes (113; 127). It is well established that CaMK phosphorylates HDACs; an event which induces the export of the deacetylase to the cytoplasm (57; 206) and tips the balance to favour HAT activity in the nucleus. In the absence of bound HDAC5, it can be speculated that HATs such as p300 would bind to MEF2, perhaps facilitated by the transcriptional co-activator PGC-1 α (130; 161). p300 increase MEF2 transcriptional activity (32; 180) by acetylating MEF2, which increases its DNA binding activity (115). p300 also acetylates histones in gene promoters which causes local chromatin relaxation and increases the access of transcription factors to their binding domains (147). As discussed in section 1.6, there is strong evidence that the *Glut4* promoter is repressed by HDAC5 (31; 123; 186). Furthermore, during exercise HDACs dissociate from MEF2 and are exported from the nucleus (122). Therefore, the observation that KN93 markedly reduced the exercise-induced increase in MEF2A binding (Figure. 5.3) and histone hyper-acetylation (Figure 5.4), supports the hypothesis that CaMK increases GLUT4 expression during exercise by initiating events leading to the export of HDAC5 from the nucleus, thus liberating MEF2 to associate with HATs, which remodel chromatin in the *Glut4* promoter. To investigate this hypothesis, research should aim to identify potential HATs that might acetylate histones in the *Glut4* promoter in response to CaMK activity and exercise.

KN93 reduced the exercise-induced increases in GLUT4 mRNA and protein levels (Figure 5.2A and 5.2B), suggesting that CaMK plays an important role in GLUT4 mRNA and protein expression during exercise. It is possible that with a larger sample size, the difference observed between the control rats and those exercised with KN93 would reach significance, suggesting that KN93 may not completely block the exercise-induced increase in GLUT4 mRNA and protein expression. This observation would not be surprising as there are additional signals besides CaMK that can also increase GLUT4 expression. Many studies have shown that the *Glut4* promoter is also regulated by another transcription factor, GEF (96; 122) and that exercise increases the binding of this factor to its *cis*-element on the *Glut4* gene (122). Other signals which regulate this transcription factor may therefore also increase GLUT4 expression. AMPK for example, which increases GLUT4 expression, also phosphorylates GEF in rat skeletal muscle and causes GEF to translocate to the nucleus and bind to its domain in the *Glut4* promoter *in vitro* (71). AMPK is activated by the drop in energy status of a muscle cell and is therefore activated during exercise of moderate to high intensity (19; 163; 187; 217). Although AMPK activity has been shown to increase GLUT4 expression, ablation of AMPK activity in transgenic animals does

not prevent the increase in GLUT4 after exercise, indicating that it is not necessary for exercise-induced expression of GLUT4 (70; 87; 168). This however does not exclude the possibility that AMPK may increase GLUT4 expression by reinforcing the CaMK signal at higher exercise intensities. As mentioned previously in section 1.9, AMPK is also activated in isolated soleus muscles during tetanic contractions (86; 222). KN93 prevented AMPK activation in these muscles in response to brief, low intensity contractions but not during contractions of higher intensity or longer duration. The effect of KN93 on AMPK activity in exercised muscles is unknown, however, it is unlikely that KN93 significantly inhibited muscle AMPK activity in the present study because of the relatively high intensity and duration of the exercise, which would likely be sufficient to activate AMPK through an AMP-dependent pathway.

Rose *et al.* (174) demonstrated that autonomous CaMK II activity was markedly increased in human skeletal muscle by ~10-fold at the onset of a bout of exercise, is then reduced to 2-3-fold above controls after 10 minutes and remains elevated at that level for the duration of a 60 minute exercise bout. Although the large increase in CaMK II activity at the onset of exercise is transient, its effects on target proteins persist throughout the exercise. Phosphorylation of phospholamban and serum response factor for example, which are targets of CaMK II, were elevated ~5 fold within 10 minutes of the exercise and remained elevated for the duration of the bout (174). CaMK II activity at the onset of exercise was not measured in this thesis, however autonomous CaMK II activity in rats was 1-7 fold higher than controls after 100 minutes of intermittent exercise (Figure 1.5A). The binding sites on CaMK II for inhibitors such as KN93 are unknown, but they appear to compete with Ca^{2+} /CaM for binding, thereby preventing the new activation and phosphorylation of CaMK II (190; 199). This is consistent with the findings in this chapter that the increases in autonomous CaMK II activity and phosphorylation at Thr 286, immediately after exercise were prevented by KN93 (Figure 5.1 C, E).

As discussed in section 1.7, it has been reported that KN93 also inhibits voltage-dependent K^+ currents (166) and L-type calcium channel activity (48) in addition to CaMK II. Although Mu *et al.* (135) recently demonstrated that KN93 inhibited MEF2 transcriptional activity in muscle by inhibiting CaMK activity and not through these other secondary effects, a weakness of the current investigation is that the specificity of KN93 was not controlled for. Therefore, some of the experiments reported in this chapter are currently being repeated with the addition of a fourth group of rats that will be injected with KN92 (an analogue of KN93 that inhibits voltage-dependent K^+ currents and L-type calcium channel activity but has no influence on CaMK II

activity), prior to exercise. It is anticipated that KN92 will not decrease the exercise induced increase in GLUT4 expression.

In summary, results from experiments in this chapter show for the first time that exercise induces a hyper-acetylation of the histones in the *Glut4* promoter in rats. Another novel finding of this chapter is that CaMK is required for the exercise induced increase in MEF2A binding to its *cis*-element in the *Glut4* promoter, hyper-acetylation of histones at this site and enhanced GLUT4 protein expression. The data suggest that CaMK activation promotes *Glut4* gene expression by increasing the accessibility of MEF2A to its binding domain on the gene.

University of Cape Town

CHAPTER SIX

Development of recombinant adenoviruses and cell culture techniques.

6.1 Introduction

In chapter 5 it was demonstrated that CaMK activity was required for exercise-induced GLUT4 expression in rat skeletal muscle. To investigate whether CaMK activation is sufficient to increase GLUT4 expression, recombinant adenoviruses that contain constructs of the human CaMK IV gene were developed to express CA or DN CaMK IV proteins in C2C12 myotubes. This chapter gives a background to adenoviral technology (section 6.2) and outlines the methods used to produce these recombinant adenoviruses. More specifically it describes the recombinant CaMK IV constructs that were inserted into the adenoviral vectors (section 6.3.1) and details the methods used to amplify the recombinant vectors (section 6.4) and produce live viruses (section 6.5). Evidence is provided that the correct CaMK IV mRNA and proteins were expressed in C2C12 myotubes and that the expressed CA CaMK IV was functional (section 6.7.1).

6.2 Background to adenoviral technology

Recombinant adenoviruses are a versatile tool for expressing transgenic proteins in a wide variety of cell types (67). Transgenes (e.g. human CaMK IV) of up to 10 kb that are under the control of a cytomegalovirus (CMV) promoter, are first cloned into a shuttle vector (e.g. pAdTrack-CMV), which is then co-transformed into *E. coli* cells with a vector that contains adenoviral backbone DNA (e.g. pAdEasy-1) as seen in Figure 6.1. Most adenoviral vectors used for transgene delivery, contain an E1 gene deletion, which renders the virus unable to replicate and form viral particles in normal cellular hosts (12). Inside the *E. coli* cells, the 2 vectors undergo homologous recombination such that the transgene of interest, a green fluorescent protein (GFP) gene and a kanamycin resistant gene are cloned into the adenoviral vector. The recombinant vectors are then transfected into packaging cells such as human embryonic kidney (HEK) 293 cells, which express the E1 adenoviral gene and allows the virus to replicate and produce viral particles, which can be isolated and stored (2; 53). Live viruses can be amplified by re-infecting the virus into 293 cells or it can infect a wide variety of other cell types, including C2C12 myotubes, to express the recombinant proteins (188).

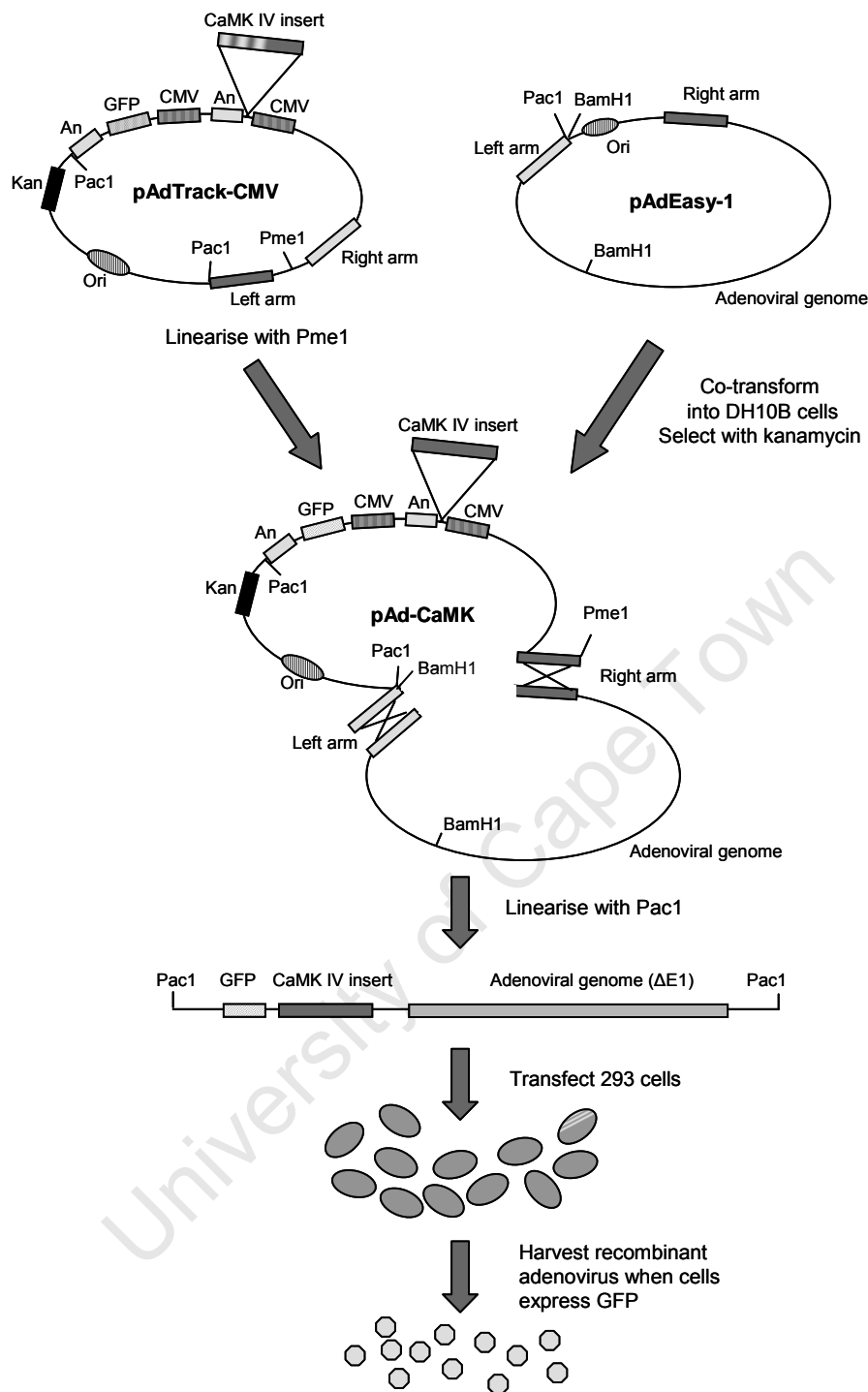


Figure 6.1. Schematic outline of the AdEasy system of adenoviral production. The CaMK IV constructs are cloned into the pAdtrack-CMV shuttle vector. The resultant vector is linearised with *PmeI* and co-transformed into bacteria with the pAdEasy-1 vector that contains adenoviral backbone DNA. Homologous recombination joins the two plasmids at the ‘left’ and ‘right’ arms and recombinants are selected for by kanamycin resistance and confirmed by endonuclease digestion. The linearised recombinant vectors are transfected into 293 packaging cells, monitored for GFP expression and live virus particles are harvested. Diagram was adapted from He *et al.* (67). CMV = cytomegalovirus promoter; GFP = green fluorescent protein gene; An = polyadenylation site; Kan = kanamycin resistant gene; Ori = origin of replication.

6.3 Production of pAd-CaMK IV vector

6.3.1 Description of the flag-tagged CaMK IV constructs: Adenoviral vectors that over express constructs of the human CaMK IV gene have been previously developed in D Kelly's Laboratory by Dr Edward Ojuka at Washington University School of Medicine following the AdEasy system of adenoviral production described by He *et al.* (67). These constructs consist of the Wild Type (WT) human CaMK IV gene; the human CaMK IV with a point mutation in the ATP binding domain (Thr 200 to Ala) causing expression of a DN CaMK IV or a truncated form of the human CaMK IV gene which expresses only amino acids (aa) 1-317 and lacks the auto-inhibitory domain, thereby producing a CA CaMK IV. As discussed in section 1.8, Chatila *et al.* (18) have demonstrated that this CA human CaMK IV protein is active in skeletal muscle even in the absence of Ca^{2+} /CaM. Conversely, the DN human CaMK IV protein is inactive even in the presence of Ca^{2+} /CaM. A schematic diagram of the proteins produced by these constructs is shown in Figure 6.2. Each construct is preceded by a sequence that codes for an eight amino acid flag-tag at the N-terminus of the protein.

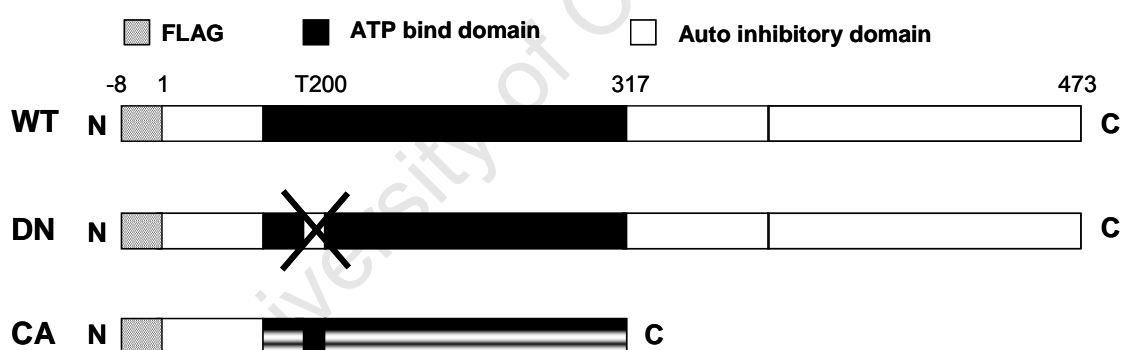


Figure 6.2. Schematic diagram of the proteins produced by the wild type (WT), dominant negative (DN) and constitutively active (CA) recombinant human CaMK IV constructs. Numbers indicate amino acid residues. FLAG = 8 amino acid Flag tag; N = amino terminus; C = carboxyl terminus.

6.3.2 Production of adenoviral vectors containing the recombinant CaMK IV constructs (pAd-CaMK vectors): The CaMK IV constructs were cloned into the pAdTrack-CMV shuttle vector purchased from Life Technologies (USA). An additional vector (CON) that did not contain any insert was included to control for effects of the adenoviral infection. As seen in Figure 6.1, the pAdTrack-CMV vector contains a kanamycin resistant gene; an origin of replication; a cytomegalovirus (CMV) promoter; a polyadenylation site; a linker between the CMV promoter

and the polyadenylation site for insertion of exogenous transgenes; and an enhanced green fluorescent protein (GFP) gene (under the control of a CMV promoter and followed by a polyadenylation site) for tracking of recombinant adenoviruses. The vector also has two adenoviral sequences that mediate homologous recombination with adenoviral vectors (e.g. AdEasy-1). The resultant pAdTrack-CMV vector containing a CaMK IV gene was linearised by digestion with the restriction endonuclease *PmeI*, purified by phenol/chloroform extraction and ethanol precipitated. The purified pAdTrack-CMV from the digest was subsequently co-transformed with the pAdEasy-1 vector, which contains the adenoviral genome, into electrocompetent *E.coli* BJ5183 cells to facilitate homologous recombination between the 2 vectors (Figure 6.1). Electroporation was performed in 2.0 mm cuvettes at 2500 v, 200 Ω , and 25 μ F in a Gene Pulser electroporator (Bio-Rad). The cells were immediately added to Luria-broth (L-broth) (Life Technologies, USA) and grown at 37°C for 20 minutes. The cell suspension (100-500 μ l) was inoculated onto 10 cm Luria-agar plates containing 50 μ g.ml⁻¹ kanamycin and incubated for 16-18 hours at 37°C. Colonies were picked and inoculated into 2 ml L-Broth with 50 μ g.ml⁻¹ kanamycin and grown for 16 to 18 hours at 37 °C. Plasmid minipreps (to isolate the vectors) were performed on the cultures using the alkali lysis method to confirm positive transformants and to obtain recombinant adenoviral vector DNA as described later in section 6.4.3. These three vectors referred to as CA-, DN- or WT- pAd-CaMK IV and a vector containing no insert called pAd-CON, were kindly donated by Prof. D. Kelly of Washington University School of Medicine.

6.3.3 The pAd CaMK IV vectors that were received were verified: Upon receipt of the original pAd-CON and 3 pAd-CaMK IV vectors from Washington University, they were digested with *PacI* or *BamHI* to confirm that they produced fragments of expected sizes. One μ g of vector was digested with 1 U. μ l⁻¹ *PacI* enzyme (NEB) (in a buffer consisting of 1X NEBuffer 1 (NEB) and 1 μ g. μ l⁻¹ BSA) or with 0.25 U. μ l⁻¹ *BamHI* enzyme (Promega) (in a buffer consisting of 1X Buffer E (Promega) and 0.25 μ g. μ l⁻¹ BSA) in 10 μ l reactions for 1 hour. The resultant fragments were resolved on 0.8% agarose gels by electrophoresis (Figure 6.3A). Two bands were observed in all undigested plasmids, one migrating at ~35 kb and that other at ~50 kb. According to the manufacturers of the Ad-Easy system, these bands represent the linear and supercoiled forms of the plasmid, respectively (67). *PacI* digestion produced fragments at ~ 35 kb for all vectors and a fragment at either 4.5 kb for pAd-CON and WT-pAd-CaMK IV or 3 kb for CA-pAd-CaMK IV and DN pAd-CaMK IV. The presence of the 3 or 4.5 kb fragment depends on whether recombination occurred between the right or left homologous regions (67). These fragment sizes

match those observed by Dr E. Ojuka at Washington University when he produced pAd-CaMK IV vectors.

*Bam*HI digestion of the original pAd-CON vector and 3 pAd-CaMK IV vectors produced 2 and 3 bands respectively as shown in Figure 6.3B. These results are consistent with those observed by He *et al.* (67) and indicate that there is an additional *Bam*HI site in the CaMK IV constructs. The *Bam*HI digest patterns varied between the 3 pAd-CaMK IV vectors because of the size and position of the insert and proved to be a useful tool to differentiate between the different vectors during subsequent procedures. In summary, bands of expected sizes were observed when the original pAd-CaMK IV and pAd-CON vectors were digested with *Bam*HI or *Pac*I indicating that the correct pAd CaMK IV vectors were received and they did not suffer degradation during transport.

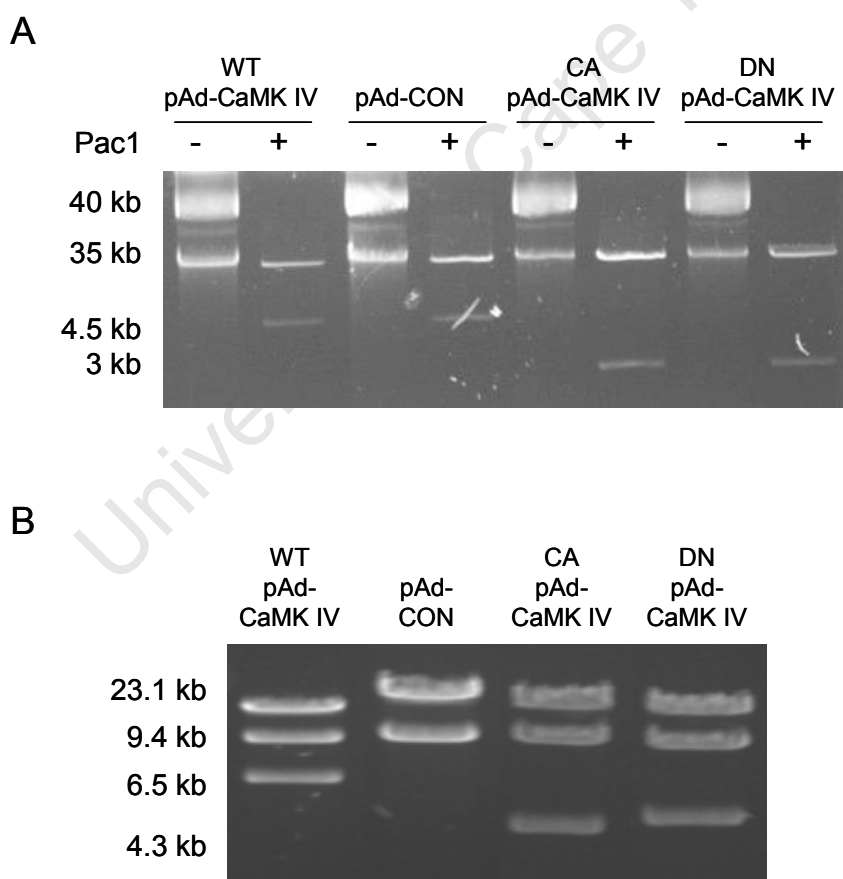


Figure 6.3. The recombinant vectors produced restriction fragments of expected sizes. 0.8% agarose gel electrophoresis of the original WT-, CA- and DN- pAd-CaMK IV and pAd-CON vectors that were received from Washington University before (-) and after (+) digestion with *Pac*I (A) or after digestion with *Bam*HI (B).

6.4 Amplification of the Ad-CaMK IV vectors

The pAd-CaMK IV and pAd CON vectors were amplified to obtain sufficient quantities for transfection experiments and for storage of plasmid stocks. To do this, vector DNA was transformed into competent *E. coli* DH10B cells and positive transformants were confirmed by performing plasmid mini-preps, digesting the vectors with *Bam*HI and visualising the digests on 0.8% agarose gels as described below. Positive transformants were used to amplify the plasmids using a maxi-prep kit (Qiagen).

6.4.1 Production of competent DH10B cells: DH10B cells from a glycerol stock were loop inoculated into 5 ml of L-broth and grown overnight at 37°C with shaking. Five hundred µl of this culture was used to inoculate 40 ml of L-broth in a 200ml flask. The culture was grown with vigorous shaking at 37°C until an optical density (OD) at 600 nm of between 0.3 and 0.4 was reached. The cells were pelleted by centrifugation at 3000 x g for 5 minutes at room temperature, gently resuspended in 20 ml of ice-cold 50 mM CaCl₂ and incubated on ice for 30 minutes. The cells were re-centrifuged at 3000 x g for 5 minutes at 4°C and resuspended in 4 ml ice-cold 50 mM CaCl₂. Competent cells were either used immediately or snap-frozen in liquid nitrogen and stored at -80 °C.

6.4.2 Transforming the adenoviral vectors into competent DH10B cells: Two hundred µl of thawed competent cells were mixed gently with 1 µg of supercoiled adenoviral vector DNA in a sterile 1.5 ml plastic tube and incubated on ice for 30 minutes. The mixture was heat shocked for 2 minutes at 42°C and 1 ml of pre-warmed L-broth was added to the cells, which were then incubated at 37°C for 45 minutes. Twenty, 50, 100 and 200 µl aliquots were plated on L-agar plates, which contained kanamycin to select for positive transformations. Transformations of pSV-βgal vector into similar DH10B cells produced about 10⁵ to 10⁶ positive transformants per µg plasmid DNA and served as a positive control for transformation efficiency of DH10B cells.

6.4.3 Diagnostic plasmid mini-preps to confirm positive transformations: Positive transformants picked from single kanamycin resistant colonies were inoculated into 5 ml of L-broth supplemented with kanamycin and grown at 37°C overnight with shaking. Cells from 1.5 ml of this culture were collected by centrifugation at 12000 x g for 1 minute at 4°C. The pellet was resuspended in 100 µl of GTE solution (50 mM Glucose; 25 mM Tris, pH 8; 10 mM EDTA) and incubated for 5 minutes at room temperature. The preparation was mixed with 200 µl of freshly made solution containing 0.2 M NaOH and 1% SDS and incubated for 5 minutes on ice before

150 μ l of 5 M potassium acetate (pH 4.8) was added. The chromosomal DNA and cellular debris was pelleted by centrifugation at 12000 x g for 5 minutes at 4°C and the supernatant transferred to a fresh tube. Plasmid DNA was ethanol precipitated at -20°C for 1 hour and resuspended in 20 μ l TE buffer and 1 μ l RNase A. Plasmid preparations were digested with *Bam*HI as described in section 6.3.3 and separated on a 0.8% agarose gel by electrophoresis, adjacent to *Bam*HI digests of the original vectors that were received from Washington University (Figure 6.4). *Bam*HI digestion of the amplified vectors produced a banding pattern identical to the original pAd-CaMK vectors confirming that the vectors were transformed into DH10B cells correctly.

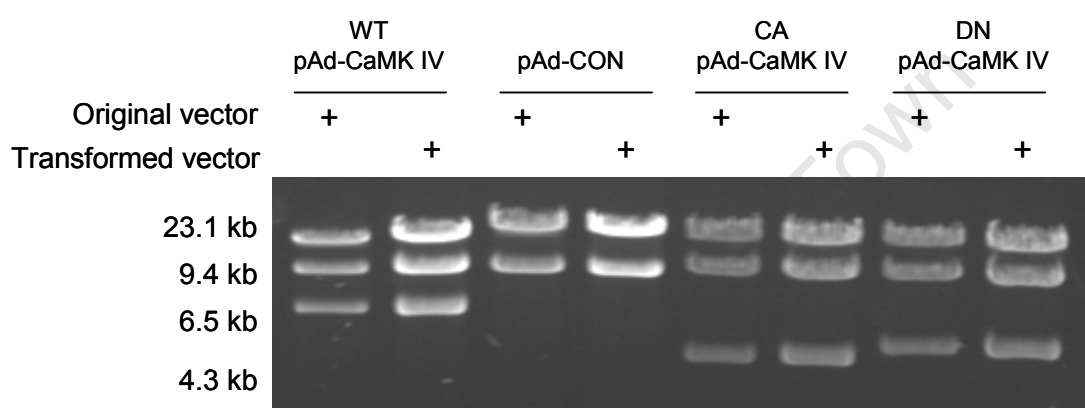


Figure 6.4. The transformed recombinant vectors produced restriction digest fragments of expected sizes. pAd-CaMK IV and pAd-CON vectors that were received from Washington University (Original vector) and vectors that were transformed into DH10B cells and isolated using mini-preps as described in section 6.4.2 (transformed vector), were digested with *Bam*HI and separated on a 0.8% agarose gel by electrophoresis.

6.4.4 Plasmid maxi-preps were performed to amplify the adenoviral vectors: Large scale preparations of recombinant CA-, DN-, and WT- pAd-CaMK IV and pAd CON plasmids were performed using the QIAGEN Plasmid Maxi kit according to the manufacturers directions. Briefly, a single DH10B colony that was confirmed to contain an adenoviral vector was inoculated into 5 ml of L-broth supplemented with kanamycin and grown at 37°C overnight with shaking. Five hundred μ l of this culture was inoculated into 500 ml L-broth supplemented with kanamycin and grown at 37°C overnight with shaking. The cells were collected by spinning at 6000 x g for 15 minutes at 4°C and sequentially resuspended in buffers supplied by the kit manufacturers to lyse the cells and precipitate genomic DNA, proteins and cellular debris. The preparation was centrifuged at 17000 rpm for 30 minutes and 14000 rpm for 15 minutes using a

Beckman JA20 rotor. The supernatant, containing plasmid DNA, was applied to a QIAGEN filter tip that was equilibrated with buffer to ensure that the plasmid DNA binds to the resin filter. RNA, proteins, dyes and low molecular weight impurities were removed by a medium salt wash and plasmid DNA was eluted in a high salt buffer. The plasmid DNA was precipitated by adding isopropanol to a concentration of 40% and collected by centrifugation at 11000 rpm in a Beckman JA20 rotor for 30 minutes at 4°C. The pellet was washed in 70% ethanol, air-dried and resuspended in 200 µl of TE buffer. The amplified recombinant adenoviral vector was digested with *Bam*HI and separated by agarose gel electrophoresis as described in section 6.3.3. The *Bam*HI digestion patterns of the amplified plasmids matched those of the original pAd-CaMK IV and pAd-CON vectors (data not shown).

6.5 Production of recombinant adenoviruses

Live recombinant adenoviruses were produced by transfecting the linearised pAd-CaMK IV and pAd-CON vectors into HEK 293 cells which express the E1 gene necessary for production of viral particles (Figure 6.1):

6.5.1 Growing 293 cells: 293 cells were grown in T-25 or T-75 flasks in Dulbecco's Modified Eagles Medium (DMEM) containing 5 mM glucose, 100 µU.ml⁻¹ streptomycin, 100 µU.ml⁻¹ penicillin, 25 µg.ml⁻¹ fungizone and 10% foetal bovine serum (FBS) at 37°C in an atmosphere of 5% CO₂/ 95% O₂. Medium was changed every 4 days and sub-confluent cultures were passaged by lifting the cells with 0.25% trypsin/ EDTA and re-plating them in 4-6 flasks as before.

6.5.2 Vector DNA was linearised with PacI: 20 µg pAd-CaMK IV and pAd CON vector DNA was digested with *PacI* as described in section 6.3.3 in a reaction volume of 50 µl for 1 hour. To remove restriction enzymes, DNA from restriction digests was made up to a volume of 100 µl using distilled water and 100 µl of chloroform isoamyl-alcohol (24:1) was added. The solution was shaken for 5 minutes and centrifuged at 13 000 x g for 5 minutes. The aqueous phase was transferred to a new tube and a volume of 100% ethanol equal to 2.5 times that of the aqueous phase was added. The DNA was precipitated overnight at -20°C and pelleted by centrifugation for 10 minutes at 13000 x g, washed with 70% ethanol, air dried and resuspended in 20 µl TE buffer. The DNA concentration of the plasmid preparations were measured using Nanodrop ND-1000 machine (Nanodrop Technology).

6.5.3 Transfecting vectors into HEK 293 cells: Recombinant adenoviral vectors were transfected into 293 cells using Lipofectamine as follows: 293 cells were plated at high density (approximately $1-2 \times 10^6$ cells) in 25 cm^2 flasks to obtain a confluency of 70-90% within 24 hours. Twenty four hours after plating, the cells were washed once with serum free DMEM and incubated in 2.5 ml Optimem I medium. A transfection mixture of $4 \mu\text{g}$ *PacI* digested adenoviral vector DNA, $20 \mu\text{l}$ Lipofectamine and $500 \mu\text{l}$ of Optimem I Medium was incubated at 25°C for 25 minutes before being added to the flasks. After incubation at 37°C for 6 hours, the medium was changed to growth medium consisting of DMEM with 10% FBS. Transfection was monitored daily by the expression of GFP.

6.5.4 Collection of viruses: When more than 90% of cells expressed GFP or more than half had lifted from the plates, the cells were scraped into 15 ml plastic tubes and pelleted by centrifugation at $2000 \times g$ for 10 minutes. Cells were resuspended in 1 ml PBS and disrupted to release the viruses by 5 x freeze-thaw cycles in an Ethanol-Dry ice and a 37°C water bath, respectively. Cellular debris was pelleted by centrifugation at $2000 \times g$ for 2 minutes and $10 \mu\text{l}$ of this supernatant was used to re-infect 20 x T75 flasks of 293 cells that had been cultured for 24 hours. The adenoviruses were harvested as before and mixed with an equal volume of 2 x viral storage buffer (10 mM Tris, pH 8; 100 mM NaCl; 0.1% BSA; 50% glycerol) and aliquots were stored at -80°C . Recombinant adenoviruses that contained WT-, CA- and DN-pAd-CaMK IV and pAd-CON vectors will be referred to as WT-CaMK IV, CA-CaMK IV, DN-CaMK IV and CON viruses, respectively.

6.6 Expression of recombinant CaMK IV proteins in C2C12 myotubes

6.6.1 Differentiation of C2C12 myotubes: Experiments were performed in C2C12 myotubes, a readily available mouse cell line that displays similar characteristics to the L6 myotubes from rats. C2C12 myoblasts were maintained on 100 mm tissue culture dishes in DMEM containing 5 mM glucose, 10 mM creatine, $100 \mu\text{U}\cdot\text{ml}^{-1}$ streptomycin, $100 \mu\text{U}\cdot\text{ml}^{-1}$ penicillin, $25 \mu\text{g}\cdot\text{ml}^{-1}$ fungizone and 10% FBS at 37°C in an atmosphere of 5% CO_2 / 95% O_2 . Medium was changed every two days and myoblasts were passaged by trypsinization with 0.25% trypsin/EDTA when cells were ~ 60% confluent. To produce differentiated myotubes, myoblasts were grown on collagen-coated 6-well plates or 100mm tissue culture dishes as described above. When myoblasts were ~80-90% confluent, the FBS in the DMEM was replaced with 2% horse

serum (HS). DMEM with 2% HS was replaced every 2 days and treatments were commenced 5-8 days later when most of the myoblasts had fused to form myotubes.

6.6.2 Infection of C2C12 myotubes with recombinant adenoviruses: Myotubes were given fresh DMEM + 2% HS and infected with a serial dilution of virus and monitored for GFP expression using a florescent microscope. The lowest quantity of adenovirus that ensured that ~ 90% of myotubes expressed GFP two days after infection was used to calculate the dose to infect myotubes grown on 100 mm collagen coated plates for subsequent experiments.

6.7 Confirmation of correct recombinant CaMK IV mRNA and protein expression

6.7.1 Analysis of recombinant hCaMK IV mRNAs in C2C12 myotubes: RNA was isolated from cells that had been infected with CA-CaMK IV, DN-CaMK IV or CON viruses using Tri reagent according to the manufacturer's instructions (Ambion). Briefly, infected C2C12 myotubes were washed twice in PBS, scrapped into 1 ml of PBS and pelleted by gentle centrifugation at 2000 x g for 2 minutes. Cells were resuspended in 1ml of Tri reagent, vortexed briefly and incubated on ice for 15 minutes. Samples were mixed with 200 µl chloroform, vortexed for 15 seconds and incubated for 15 minutes at room temperature. Homogenates were centrifuged at 12000 x g for 15 minutes at 4°C and RNA from the aqueous phase was precipitated overnight at -20°C with an equal volume of isopropanol. The RNA was pelleted by centrifugation at 12000 x g for 10 minutes, washed in 75% ethanol, air-dried and resuspended in 20 µl DEPC treated water. RNA concentration and quality were determined and cDNA was synthesised from 1 µg RNA as described in section 3.8.2 and 3.8.3. Conventional PCR was performed using cDNA generated from C2C12 myotubes that were infected with the CA- or DN-CaMK IV or CON viruses using primers which amplify a region corresponding to aa 399-466 near the carboxyl (C) terminus of the human CaMK IV gene that is present in the DN but not in the truncated CA-CaMK IV construct. Another set of primers was used to amplify a region near the amino (N) terminus of human CaMK IV corresponding to aa 40-103 that is present in both CA and DN constructs. The positions of the primers on the corresponding CaMK IV proteins are indicated by the arrows in Figure 6.5A and the primer details are in Table 3.4. Figure 6.5B shows that cells infected with the DN-CaMK IV virus, expressed human CaMK IV mRNA that contained both the C- and N-terminal regions whereas cells infected with the CA-CaMK IV virus only expressed mRNA only from the N-terminal region. As expected no human CaMK IV transcripts were present in cells infected with the CON virus that does not contain a human CaMK IV gene and the human CaMK IV primers

did not amplify products from endogenous C2C12 mouse genomic DNA. These data shows that the CA- and DN-CaMK IV viruses expressed the correct CaMK IV transcripts and that the CON virus was a suitable negative control.

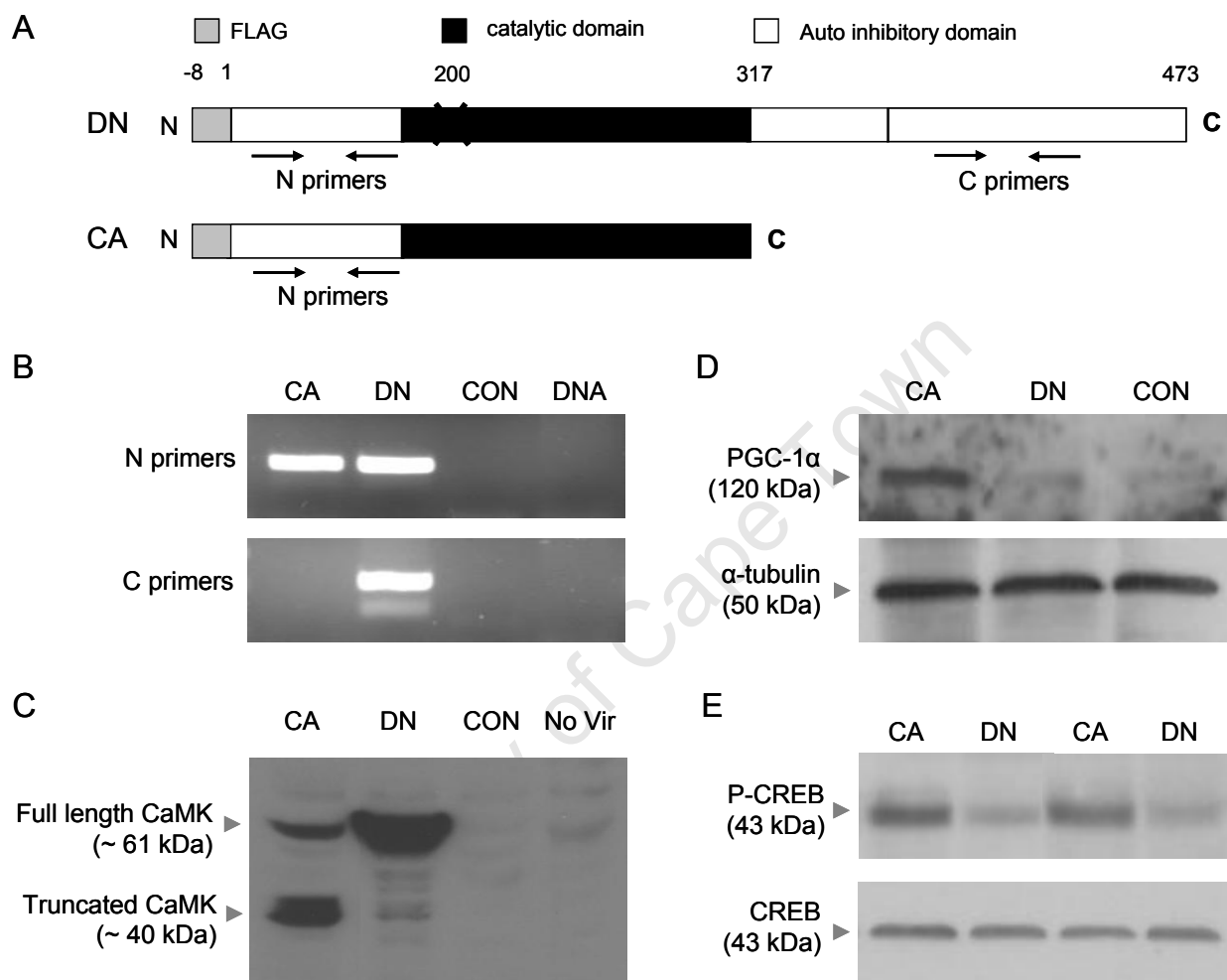


Figure 6.5. Recombinant adenoviruses express functional CaMK proteins in C2C12 myotubes. C2C12 myotubes were infected with CA-CaMK IV or DN-CaMK IV or CON viruses. The CA and DN CaMK IV viruses contain genes that express CA or DN CaMK IV proteins that are preceded by an 8 aa Flag tag (illustrated in A). The CON virus does not contain an inserted CaMK IV gene. B. PCRs using primers which amplify regions that correspond to the amino (N) and carboxyl (C) termini of human CaMK IV (indicated by the arrows in A) were performed on cDNA from infected myotubes. PCR using C2C12 genomic DNA (DNA) is shown to control for cross-reactivity of the primers with the mouse genome. B. Representative western blots from infected myotubes using antibodies that recognise FLAG tag (C), PGC-1 α (D) or Phospho-CREB (E). α -Tubulin (D) and CREB (E) blots of the same samples are included as internal controls.

6.7.2. *CA and DN CaMK IV viruses express functional recombinant CaMK IV proteins in C2C12 myotubes.* The recombinant CaMK IV proteins are preceded by an 8 aa flag tag. Therefore the content of FLAG tagged proteins in C2C12 cells that were infected with CA- and DN-CaMK IV viruses was measured by western blot to check that recombinant proteins of expected sizes were expressed. Myotubes were washed twice with PBS and were scraped into 1 ml of HES or 'Phospho' buffer (section 3.5). The proteins were homogenised using a glass homogeniser attached to an electric drill press set at slow speed, solubilised in Laemmli sample buffer and assayed by Western blot using an anti-FLAG antibody (Table 3.3) as described in section 3.6.1. In the cells infected with the CA-CaMK IV virus, which contains a truncated CA-CaMK IV gene, the Flag tagged proteins migrated faster (~ 40 kDa) than those from cells infected with the DN-CaMK IV viruses (~ 61 kDa), which contains the full length DN-CaMK IV gene (Figure 6.5 C). No Flag tag was detected in cells infected with a virus that did not contain a CaMK IV gene (CON) or in cells that were not infected with a recombinant adenovirus. These results indicate that recombinant CA- and DN-CaMK IV proteins of expected sizes were expressed. Activation of CaMK IV has been shown to increase the expression of PGC-1 α and to phosphorylate CREB (39; 60; 63; 225). Western blots show that more PGC-1 α and phosphorylated CREB were present in C2C12 cells expressing the CA CaMK IV compared to the DN form (Figure 6.5 D and E). These results provide strong evidence that the correct recombinant CaMK IV proteins were expressed in C2C12 myotubes and that the CA CaMK IV was more effective at phosphorylating known substrates compared to the DN CaMK IV.

6.8 Source of Materials used.

DH10B and HEK 293 cells were a gift from Assoc. Prof. Arie Katz from the University of Cape Town (Cape Town, South Africa). Cell culture materials were purchased from Highveld Biological (Johannesburg, South Africa) and C2C12 myotubes were from American Type Culture Collection (Mannassas, VA). The source of the antibodies are detailed in Table 3.3. Plasmid maxi-preparation kit (Qiagen) was purchased from Southern Cross Biotechnology (Cape Town, South Africa). Lipofectamine (Invitrogen) was purchased from Scientific Group (Johannesburg, South Africa). *PacI* was purchased from New England Biolabs (Ipswich, MA) and *BamHI* (Promega) was purchased from Whitehead Scientific (Cape Town, South Africa). All other chemicals and materials were purchased from Sigma (St Louis, MA).

CHAPTER SEVEN

CaMK IV activation increases GLUT4 expression in C2C12 myotubes.

7.1 Introduction

In chapter 4, evidence was provided that a bout of exercise increases the binding of MEF2A to its binding domain in the *Glut4* promoter and enhances GLUT4 mRNA and protein expression in rat skeletal muscle. In chapter 5, it was demonstrated that CaMK II is activated during exercise and is necessary for the exercise-induced adaptations in GLUT4 expression. In those experiments CaMK activity was inhibited in rats by administration of KN93, 30 minutes prior to a bout of exercise. KN93 reduced the increase in GLUT4 mRNA and protein normally seen in skeletal muscle after exercise, and completely blocked the increase in MEF2A binding to the *Glut4* promoter. These experiments however, do not tell whether CaMK activation is sufficient to enhance GLUT4 expression. Exercise activates numerous other signalling intermediates such as AMPK or p38 MAPK and it is possible that these signals are also required, in addition to CaMK, to increase GLUT4 expression (213; 216). The purpose of the study presented in this chapter was to test whether activation of CaMK in skeletal muscle is sufficient to increase GLUT4 expression and MEF2A binding to the *Glut4* promoter. CA- and DN-CaMK IV proteins were therefore expressed in C2C12 myotubes (mouse) using recombinant adenoviruses. Results from these experiments show that there was more MEF2A bound to the *Glut4* promoter and higher GLUT4 mRNA and protein contents in cells expressing CA-CaMK IV, compared to DN-CaMK IV.

7.2 Methods

7.2.1 Tissue Culture. C2C12 myotubes were grown for 5 days in 100 mm collagen-coated plates until fully differentiated as described in section 6.6.1. To express CA- and DN-CaMK IV proteins, myotubes were given fresh DMEM supplemented with 2% HS and were infected with CA-CaMK IV, DN-CaMK IV or CON viruses as described in 6.6.2. Myotubes were left in culture until GFP was clearly seen in the majority of myotubes (~3 days) and MEF2A protein content, MEF2A binding to the *Glut4* promoter and GLUT4 mRNA and protein levels were assayed.

7.2.2 *Western Blotting*. GLUT4 and MEF2A protein contents in infected myotubes were measured by western blots as described previously. Briefly, C2C12 myotubes were washed twice in PBS and homogenized in HES buffer (section 6.7.2). The protein concentration was determined (section 3.5) and a quantity of protein (Table 3.3) was solubilised in Laemmli sample buffer, separated by SDS-PAGE and transferred to PVDF membranes using electrophoresis as described in section 3.6.1. MEF2A and GLUT4 protein contents on the membranes were immuno-detected as described in section 4.2.3 and were normalised to values from α -tubulin blots.

7.2.3 *Real Time quantitative PCR*. cDNA was generated from total RNA isolated from C2C12 myotubes as described in section 6.7.1. Real time PCR was performed on the cDNA templates using primers which target the mouse GLUT4, GAPDH and RS12 genes (Table 3.4) as described in section 3.8.4. Relative GLUT4 mRNA levels were calculated based on the threshold cycle of the PCR and were normalized to RS12 and GAPDH house keeping genes and expressed relative to a control in each experiment according to the $2^{-\Delta\Delta CT}$ method described by Livak *et al.* (112).

7.2.4 *Chromatin immunoprecipitation assays*. ChIP assays were performed on C2C12 myotubes using a Kit from Upstate Cell Signalling Solutions. Myotubes were crosslinked in DMEM containing 1% formaldehyde for 10 minutes at room temperature with gentle shaking. Crosslinking was stopped by the addition of 0.125 M glycine for 5 minutes. The cells were washed 3 times with PBS and scraped into 500 μ l SDS lysis buffer (see section 3.9.1). Chromatin was sonicated, pre-cleared of endogenous antibodies and used in ChIP assays with an anti-MEF2A antibody and primers which amplify the MEF2 binding domain on the mouse *Glut4* promoter (Table 3.4) as described in section 3.9.1 and 3.9.2. 1% of the sample used for each ChIP assay was also reverse cross-linked, purified and PCR amplified. Negative control experiments that omitted the antibody or used a non-specific antibody (anti-IgG) were performed in parallel and PCR was also performed on DNA from MEF2A ChIP assays using primers which span a region approximately 3 kb downstream from start site in the mouse *Glut4* gene that does not contain a MEF2 *cis*-element.

7.2.5 *Statistical analysis*. Data are presented as means \pm SD. Statistical differences between treatments were determined using a one-way ANOVA with Fisher's least significant differences post hoc test or a Student's t test as appropriate. Significance was accepted at $P < 0.05$.

7.3 Results

7.3.1 Constitutively active CaMK IV increases GLUT4 mRNA and protein levels in C2C12 myotubes. To determine whether activation of CaMK was sufficient to increase GLUT4 expression in skeletal muscle, CA- and DN- CaMK IV proteins were expressed in C2C12 myotubes as described in 7.2.1. Western blots using antibodies directed against the Flag attached to the recombinant proteins indicate that a truncated CaMK IV protein was expressed in the CA-CaMK IV cells and a full length CaMK IV protein was expressed in the DN-CaMK IV cells (Figure 6.5C). Figure 7.1 shows that both GLUT4 mRNA (A, $P = 0.001$) and protein (B, $P = 0.003$) levels were ~ 2-fold higher in cells expressing CA CaMK IV compared to those expressing DN CaMK IV, providing direct evidence that CaMK activation increases GLUT4 expression.

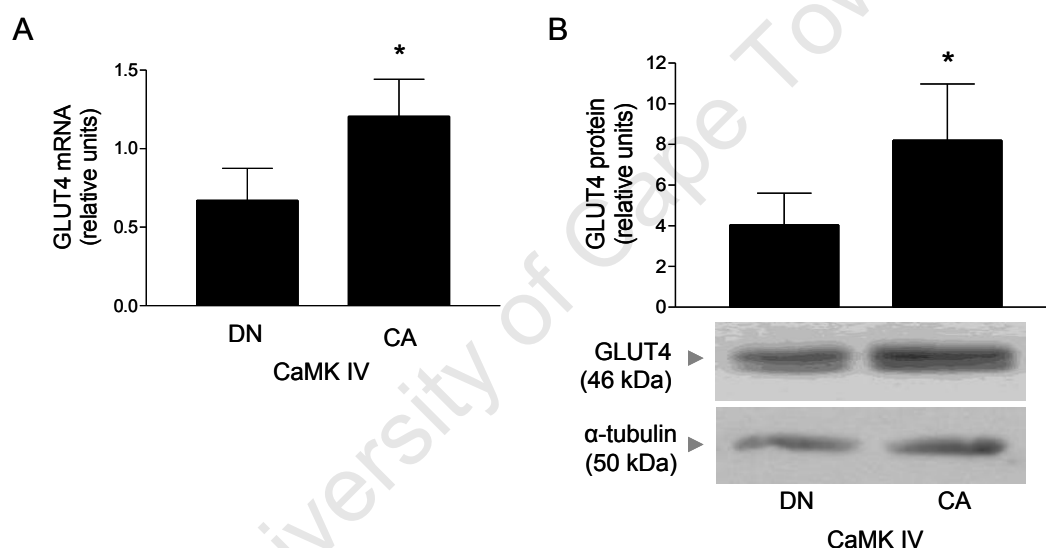


Figure 7.1. GLUT4 mRNA and protein are increased by CA CaMK IV. C2C12 myotubes were infected with adenoviruses that express CA- or DN-CaMK IV proteins. A. GLUT4 mRNA was measured in infected myotubes by quantitative RT-PCR and normalised to GAPDH mRNA. $n = 6$ independent experiments, * $P = 0.001$ vs. DN. B. GLUT4 protein was measured by western blot and normalised to α -tubulin. $n = 6$ independent experiments, * $P = 0.003$ vs. DN. Shown are representative blots for GLUT4 and α -tubulin. Data are presented as means \pm SD.

7.3.2 Constitutively active CaMK IV increases MEF2A binding to its cis-element on the *Glut4* promoter but does not increase total MEF2A content in C2C12 myotubes. To determine whether CaMK also increases the binding of MEF2A to the *Glut4* gene, ChIP assays using an anti-MEF2A antibody and primers that amplify the MEF2 binding domain in the *Glut4* promoter were performed on C2C12 cells expressing CA and DN CaMK IV proteins. Cells that were

infected with an adenovirus that does not express a recombinant CaMK IV protein were included as a control. Figure 7.2A shows that there was ~ 1.7-fold more MEF2A bound to its *cis*-element on the *Glut4* promoter in cells expressing CA CaMK IV compared to controls or cells expressing DN CaMK IV ($P < 0.001$). No PCR products were detected when ChIP assays used a non specific antibody (anti-IgG) or primers that amplify a region ~3 kb downstream from the MEF2 binding site, which does not contain a MEF2 *cis*-element (Figure 7.2A). These results ensure that the co-immunoprecipitation of DNA was specific to the MEF2A antibody and that the assay is sensitive to within 3kb of DNA from the MEF2 binding site in the mouse *Glut4* promoter. There was no difference in total MEF2A content between cells expressing a CA-, DN- or no- CaMK IV protein (Figure 7.2B, $P > 0.33$).

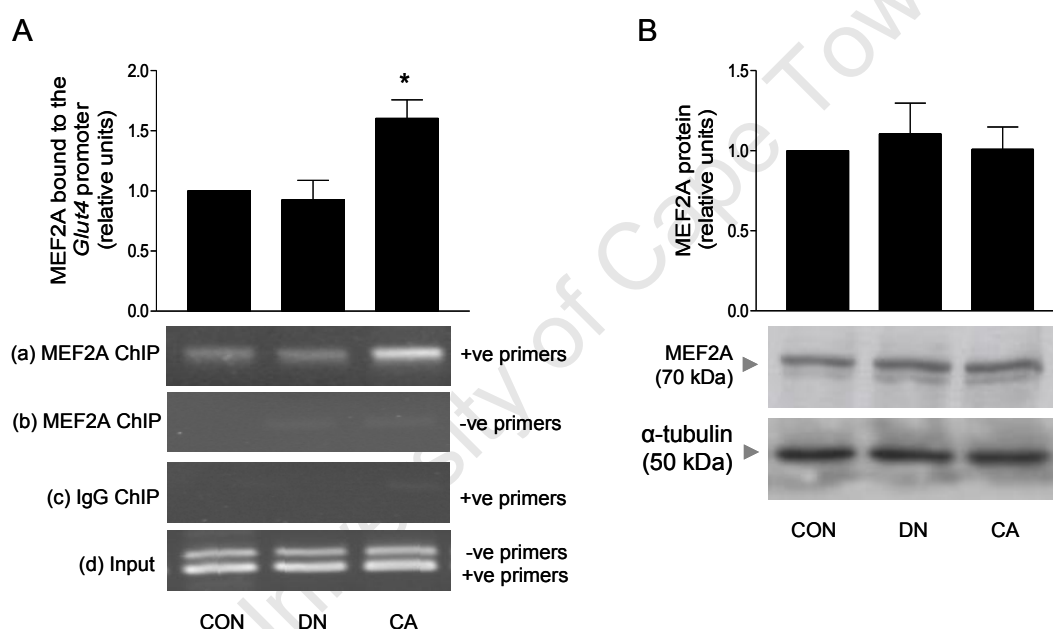


Figure 7.2. CA CaMK IV increases MEF2A binding to the *Glut4* promoter in C2C12 myotubes but does not alter total MEF2A content. C2C12 myotubes were infected with adenoviruses that express CA- or DN-CaMK IV proteins or with an adenovirus that does not express any recombinant proteins (CON). A. ChIP assays using an anti-MEF2A (a) or anti-IgG (c) antibody were performed on infected myotubes and PCR was performed on co-immunoprecipitated DNA using primers which amplify the MEF2 binding site *Glut4* promoter (+ve primers). The histogram presents MEF2A ChIP data from 4 independent experiments. * $P = 0.001$ vs. DN and CON. PCR was performed on DNA from MEF2A ChIP assays using primers that span a region in the *Glut4* gene that does not contain a MEF2 *cis*-element (-ve primers) (b). Chromatin from an aliquot of sample that did not undergo immunoprecipitation was PCR amplified using +ve and -ve primers for comparison (Input) (d). B. Representative MEF2A and α -tubulin western blots. In the histogram, signal intensities for MEF2A were normalised to α -tubulin and expressed relative to a CON in each experiment ($n=4$). Data are presented as means \pm SD.

7.4 Discussion

The main finding from the experiments in this chapter is that expression of CA CaMK IV in C2C12 myotubes results in higher levels of GLUT4 mRNA and protein and more binding of MEF2A to the *Glut4* promoter, compared to cells that express DN CaMK IV (Figure 7.1A and B and 7.2A). These results provide evidence that CaMK IV activation is sufficient to increase GLUT4 expression in mouse skeletal muscle cells. Total MEF2A levels were not different between CA and DN CaMK IV expressing cells, indicating that the enhanced binding was not likely due to increased MEF2A expression. Instead, the increased binding was possibly due to increased binding activity, translocation of MEF2 to the nucleus and/or an increase in the accessibility of MEF2 to its binding domain. Holmes *et al.* (71) recently demonstrated in rats that were injected with AICAR, that AMPK increased the nuclear translocation of MEF2 and its binding to the *Glut4* promoter in skeletal muscle *in vitro*. However, it is unclear whether translocation of MEF2A to the nucleus in response to CaMK IV activation played a part in the increased MEF2A-DNA binding in the presents study. As discussed previously in section 5.4, CaMK may also influence MEF2A binding to DNA by disrupting MEF2/HDAC5 complexes (57; 124; 206), which allows MEF2A to associate with cofactors such as PGC-1, which recruits factors having histone acetyl transferase (HAT) activity, such as p300. p300 has been shown to acetylate both MEF2, which increases its DNA binding activity, and histones within nucleosomes, which would conceivably increase accessibility of MEF2A to chromatin in gene promoters (115; 147; 227). Our laboratory is currently conducting experiments to investigate whether CaMK activity influences the nuclear translocation and acetylation of MEF2A and the acetylation of histones in the *Glut4* promoter. In section 6.7.2 it was reported that CA CaMK IV expressing C2C12 cells had more PGC-1 protein than those expressing a DN CaMK IV (Figure 6.5D) suggesting that the effect of CA CaMK IV on GLUT4 expression may also be enhanced by the elevated levels of PGC-1.

Passier *et al.* (155) have reported that CaMK signalling does not alter MEF2-DNA binding activity. Using gel mobility shift assays on cardiac muscle nuclear extracts, they found no difference in the *in vitro* binding of MEF2A to an oligonucleotide containing the MEF2 *cis*-element between wild-type mice and transgenic mice that express CA CaMK IV proteins. This observation contradicts the findings in this chapter, which shows that CaMK IV activation increases DNA binding in skeletal muscle cells *in vivo*. The conflicting observations could be attributed to the differences between the tissues (mouse cardiac muscle vs. mouse C2C12

myotubes) or DNA binding assays (EMSA vs. ChIP) used in the two studies. As discussed in section 4.4, gel mobility shift analysis is suitable for determining the extent of transcription factor interaction with DNA under *in vitro* conditions and is particularly sensitive to changes in binding caused by alterations in the nuclear abundance or intrinsic binding activity (e.g. by post translational modification) of the transcription factor. However, *in vivo* binding conditions are often difficult to recreate *in vitro*. For example, EMSA would be insensitive to any changes in chromatin structure caused by post translational modification of histones within nucleosomes, which influence access of transcription factors to their binding domains. In Chapter 5 evidence was provided that the *Glut4* gene might be regulated by chromatin remodelling when it was shown that exercise caused a hyper-acetylation of *Glut4* promoter histone H3 in a CaMK dependent manner. Furthermore, recent findings indicate that *Glut4* promoter activity is inhibited by class II histone deacetylases (123; 186). Additionally, binding of proteins which require DNA secondary structure, such as looping, to bring two distal binding sites into close proximity, or those that require multi-protein complex formation to stabilize protein-DNA interactions, may not be recreated and detected in a gel mobility shift analysis (210). In contrast, ChIP assays offer the ability to detect a protein at its *in vivo* binding site, and would therefore be sensitive to changes in binding facilitated by chromatin remodelling (152).

CaMK IV is not endogenously expressed in skeletal muscle (1; 174). In section 1.7, its use as a surrogate to study CaMK II in skeletal muscle were justified by arguing that CaMK II and CaMK IV phosphorylate common substrates, including HDAC4 (7; 234), HDAC5 (109; 124), CREB (119; 191), SRF (40; 131), ATF-1(192) and c/EBP β (209; 232). Furthermore, phosphorylation of HDACs by both CaMK II and IV leads to a dissociation of HDACs from MEF2, export of HDACs from the nucleus and an activation of MEF2 transcriptional activity (7; 109; 113; 124; 234). However the effects of over-expressing CaMK IV on other kinases that are found endogenously in skeletal muscle, such as CaMK II, are not clear. For example, over expression of CaMK IV in Hela cells has been shown to reduce the nuclear localisation of CaMK II (145). Furthermore because CaMK II is capable of phosphorylating additional residues in CREB, SRF and ATF-1 that are not phosphorylated by CaMK IV, some differences in the physiological roles between endogenously expressed CaMK II and ectopic expression of CaMK IV may exist. Therefore results in this chapter await confirmation when the technology for expressing DN and CA forms of CaMK II proteins is developed.

In summary, novel evidence has been presented that CaMK IV activation increases MEF2A binding to the *Glut4* promoter and increases GLUT4 mRNA and protein content in mouse C2C12 myotubes. These data is in line with previous findings, which suggest that CaMK II mediates the exercise-induced increase in MEF2A binding to the *Glut4* gene and GLUT4 expression.

CHAPTER EIGHT

Summary of the main findings and potential avenues for future research

The general purpose of this thesis was to investigate the role that CaMK activation plays in the exercise-induced increase in GLUT4 expression in skeletal muscle because improving GLUT4 content in skeletal muscle is a potential means of therapy for chronic diseases such as type II diabetes. More specifically, the thesis examined the influence of CaMK activity on the binding of MEF2A to its *cis*-element in the *Glut4* promoter and on the acetylation of histones near this region in response to exercise. Figure 8.1 provides a proposed mechanism for the increase in GLUT4 expression after exercise and is based on data that was presented in this thesis and from the literature.

In this thesis, ChIP assays performed on muscles from rats that were exercised by swimming, showed that the amount of MEF2A which was bound to the *Glut4* promoter *in vivo*, increased at 6 hours post-exercise (Figure 4.2A). When the normal rise in CaMK II activity in muscle was prevented by injecting rats with KN93 (Figure 5.1C), exercise failed to increase *Glut4* promoter-bound MEF2A (Figure 5.3). The pattern of MEF2A bounding to the *Glut4* promoter in these experiments, closely correlated with GLUT4 mRNA and protein levels, which were also increased after exercise, and reduced by KN93 (Figure 5.2A and B). These novel findings support the notion that increased MEF2A binding to the *Glut4* promoter after exercise regulates GLUT4 expression in a CaMK II dependent manner. Because exercise activates numerous other signals that could also potentially affect MEF2A binding and GLUT4 expression (reviewed by Ojuka *et al.* (149)), CA and DN human CaMK IV proteins were expressed in C2C12 mouse muscle cells to determine if CaMK activation was sufficient to induce GLUT4 expression. It was found that expression of CA CaMK IV was sufficient to significantly enhance MEF2A binding to the *Glut4* promoter and increase GLUT4 mRNA and protein levels, compared to expression of a DN CaMK IV (Figure 7.1 and 7.2).

It has been reported that increased binding of MEF2A to its site in gene promoters is associated with a hyper-acetylation of histones within the gene (181). In this thesis, ChIP assays revealed that exercise caused a hyper-acetylation of the histones at the MEF2A binding site in the *Glut4* gene that was largely prevented by KN93 (Figure 5.4), suggesting that this hyper-acetylation was mediated by CaMK II activity. These novel findings are consistent with a recent

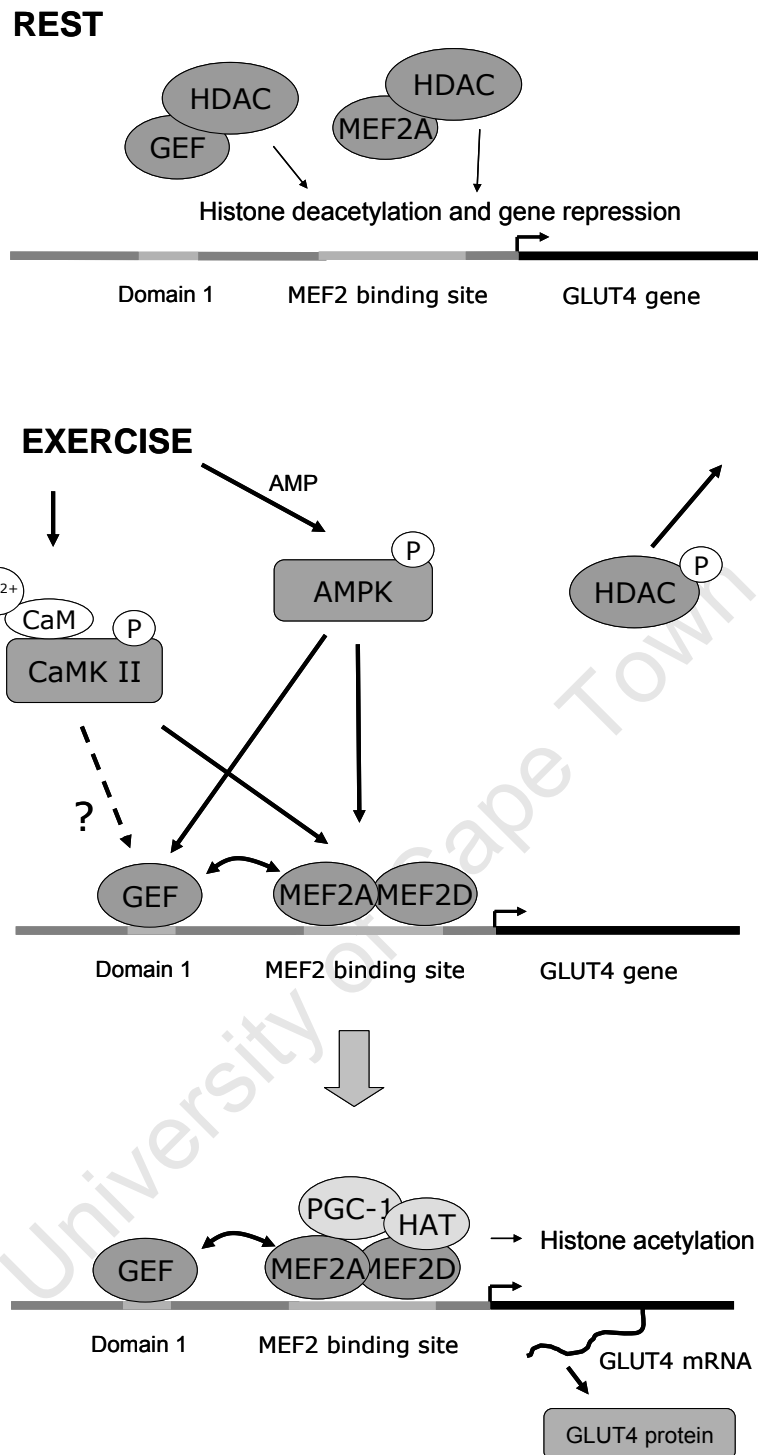


Figure 8.1. Proposed mechanism of the regulation of skeletal muscle GLUT4 expression in response to exercise. During rest, myocyte enhancer factor 2A (MEF2A) and GLUT4 enhancer factor (GEF) are bound by class II histone deacetylases (HDACs) which represses glucose transporter-4 (GLUT4) transcription by deacetylating histones that are proximal to MEF2 and GEF binding domains. During exercise, adenosine monophosphate (AMP) activated protein kinase (AMPK) and Ca²⁺/calmodulin (Cam) dependent kinase II (CaMK II) are phosphorylated (P) and activated. Activation of these kinases results in an increase in the binding of MEF2A and GEF to their binding domains in the *Glut4* promoter. Although the exact mechanism for this enhanced binding is unclear, these kinases also phosphorylate HDACs, which initiates the export of the deacetylases from the nucleus. This event facilitates MEF2A to associate with cofactors such as peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC-1 α), which recruits factors that have intrinsic acetyl transferase (HAT) activity such as p300, resulting in hyper-acetylation of the *Glut4* promoter and an increase in GLUT4 mRNA and protein.

observation by Mukwevho *et al.* (136) that activation of CaMK in C2C12 myotubes by incubation with caffeine, caused an increase in the acetylation of Histone H3 at the MEF2 site in the *Glut4* promoter. Furthermore, these authors also showed a clear correlation between the level of histone acetylation and MEF2A binding at this site (136).

Collectively, the findings from these studies suggest that during exercise, CaMK regulates GLUT4 expression by hyper-acetylating histones in the *Glut4* promoter and increasing the binding of MEF2A to its *cis*-element. The data however, does not provide a clear mechanism of how CaMK increases histone acetylation and MEF2A binding. As discussed previously throughout this thesis (section 1.5-1.7; 4.4; 5.4; 7.4), the increased binding of MEF2A to the *Glut4* promoter in response to exercise and CaMK activity may be attributed to some or all of the following factors: a) nuclear enrichment of MEF2A, b) increased intrinsic binding activity of MEF2A or c) increased accessibility of MEF2A to its binding domain. Although nuclear MEF2A content in response to exercise or CaMK activation was not measured in this thesis, it has previously been shown that exercise increases the nuclear translocation of MEF2A without affecting total MEF2A content (122). As discussed in section 1.7, there is also evidence suggesting that CaMK activity favours the binding of MEF2 factors to HATs instead of HDACs. HATs, such as p300/CBP can acetylate lysine residues in the transactivation domain of MEF2C, which increases MEF2C-DNA binding (3; 115). Because the lysine residues in MEF2C that are acetylated by p300 are conserved between MEF2A, and MEF2D in various species, this mechanism of increased binding activity is likely to be general amongst MEF2 factors (115). The increased binding of MEF2A to the *Glut4* promoter that was observed in this thesis therefore supports the hypothesis that exercise increases MEF2A binding by causing the nuclear translocation and acetylation of MEF2A, via a CaMK dependent mechanism. This hypothesis however, requires clarification that CaMK activation causes the nuclear translocation and acetylation of MEF2A.

It has been shown that p300 also acetylates histones within gene promoters that are regulated by MEF2 factors, which reduces chromatin compaction and enhances access of transcription factors to their binding sites on DNA (125; 147). In contrast HDACs remove acetyl groups from histones in gene promoters thereby reducing transcription factor access and preventing transcription. The hyper-acetylation of *Glut4* promoter histones that was observed in this thesis, supports the hypothesis that exercise remodels chromatin in the *Glut4* promoter via CaMK-mediated association of MEF2A with HATs instead of HDACs. Recently, Sparling *et al.*

(186) provided evidence that HDAC5 inhibited GLUT4 expression by associating with MEF2 and GEF transcription factors at their binding sites in the *Glut4* promoter (see section 5.4), suggesting that HDACs do not prevent MEF2A binding to the *Glut4* promoter. Therefore, although a strong correlation has been demonstrated between MEF2A binding and histone acetylation at the *Glut4* promoter (Chapter 5; (136)), it is still unclear whether histone hyper-acetylation directly facilitates increased MEF2A binding.

The finding that KN93 did not completely block the increase in GLUT4 mRNA and protein after exercise suggests that other signals which are activated by exercise might also increase GLUT4 expression. As discussed earlier in this report (section 1.9 and 5.4), this finding is consistent with evidence showing that AMPK, which is activated by exercise of moderate to high intensity, also increases GLUT4 protein in muscle (44; 71; 216). More recently, McGee *et al.* (123) demonstrated that, like CaMK (124), AMPK also regulates gene transcription through HDAC5. They showed that expression of a CA- but not a DN-AMPK in human primary muscle cells causes HDAC5 to be phosphorylated at S259 and S498, associate with the chaperone protein 14-3-3 and be exported from the nucleus (123). Furthermore they showed that CA-AMPK expression reduced the amount of HDAC5 that was associated with the *Glut4* promoter and increased GLUT4 expression (123). It is not surprising that AMPK and CaMK both phosphorylate HDACs because they have similar substrate preferences and recognise the same amino acid sequences (106; 208). These findings reinforce a previous report by Ojuka *et al.* (150) that both AMPK and CaMK regulate GLUT4 expression. However, the relative importance of CaMK and AMPK signalling during exercise has not been elucidated. Ojuka *et al.* (149; 150) hypothesised that at lower-intensity exercise, which does not activate AMPK, CaMK would be the primary signal to regulate the *Glut4* gene but that high-intensity or prolonged exercise would activate both AMPK and CaMK to induce a more robust increase in GLUT4 expression.

There is vast evidence in the literature showing that prolonged periods of physical inactivity (142) such as limb immobilisation or bed rest (13; 35) decreases GLUT4 content, which induces insulin resistance and can lead to chronic diseases such as type II diabetes (107). Cohen *et al.* (27) recently reported that reduced neural activity in muscle (e.g. by surgical denervation), resulted in a dramatic increase in HDAC4 expression, leading to a robust accumulation of the deacetylase in the nucleus. As explained throughout this thesis, an accumulation of nuclear class II HDACs would repress MEF2 dependent genes, including *Glut4*. Because physical inactivity is associated with reduced neural activity to the muscle, it can be speculated that the reduced

GLUT4 expression caused by muscle immobilisation may have been attributed to a hypo-acetylation of histones in the *Glut4* promoter resulting from the accumulation of class II HDACs in the nucleus. In contrast, physical activity improves insulin sensitivity in patients with type II diabetes primarily by increasing GLUT4 expression in skeletal muscle (reviewed by Ivy *et al.* (84)). Results from the current investigation therefore suggest that pharmaceuticals which activate CaMK II in muscle, or those that increase the acetylation of histones in the *Glut4* promoter or enhance MEF2A binding to its *cis*-element in the *Glut4* gene might ameliorate the negative impact of nuclear HDAC accumulation in muscle and improve GLUT4 levels in patients who are immobile. Such pharmacological interventions could potentially improve insulin resistance in patients with type II diabetes and prevent the onset of the disease in high risk individuals who cannot exercise because of ill health or other reasons.

In summary this research has identified CaMK II as an important signal in skeletal muscle that increases GLUT4 expression after a bout of exercise. It also provides evidence that the mechanisms whereby CaMK increases GLUT4 expression likely involve enhanced access of MEF2A to its binding domain in the *Glut4* promoter, which is facilitated by hyper-acetylation of the histones within this region.

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