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To my parents, brother and Gunilla

“It Takes a Fool to Remain Sane”

- Ola Salo (The Ark)

ABSTRACT

Obesity and type 2 diabetes are major and rapidly increasing health problems in society. They are associated with several life-threatening conditions, including heart and renal failure, and damage to the nervous system. An inability of cells to respond normally to insulin, insulin resistance, is a key feature in obesity and type 2 diabetes.

Ca^{2+} is a versatile messenger that regulates diverse cellular functions such as fertilization, electrical signaling, contraction, synaptic transmission, gene transcription, hormonal signaling, metabolism, and cell death. To exert these diverse effects, duration, amplitude and spatial distribution of Ca^{2+} need to be tightly regulated. The role of Ca^{2+} in insulin signaling under normal conditions and in association with insulin resistance is uncertain.

This thesis focuses on Ca^{2+} fluxes and insulin action in cardiac and skeletal muscles. In the first two papers we examined the effect of insulin on Ca^{2+} homeostasis in normal, freshly isolated mouse ventricular cardiomyocytes and how Ca^{2+} handling was changed in an animal model of obesity and insulin resistance, *ob/ob* mice. *Ob/ob* cardiomyocytes showed prolonged electrically evoked Ca^{2+} transients and impaired mitochondrial Ca^{2+} handling, which resulted in extra Ca^{2+} transients that may predispose for arrhythmias *in vivo*. Moreover, we observed decreased ion fluxes through canonical transient receptor potential 3 (TRPC3) channels, which may affect intracellular Ca^{2+} homeostasis and hence cellular function.

In the following two papers, we investigated the role of Ca^{2+} in insulin-mediated glucose uptake in adult skeletal muscles. Increased Ca^{2+} influx in the presence of insulin potentiated glucose uptake in muscles from both normal and *ob/ob* mice, whereas decreased Ca^{2+} influx was associated with decreased insulin-mediated glucose uptake. In addition, TRPC3 protein expression was knocked down using a novel transfection technique with small interfering RNA coupled to carbon nanotubes, which resulted in large decreases in diacylglycerol-induced Ca^{2+} influx and insulin-mediated glucose uptake. Insulin-mediated glucose uptake occurs via the glucose transporter 4 (GLUT4) that was found to co-localize with TRPC3 in the t-tubular system, which is considered to be the predominant site of glucose uptake in skeletal muscle.

Taken together, these studies shed light on how insulin and Ca^{2+} interact in signaling in cardiac and skeletal muscles. In the heart, components and channels that alter intracellular Ca^{2+} handling and might be involved in the development of acute cardiac failure in insulin resistant conditions have been identified. Further, we demonstrate that Ca^{2+} is important for insulin-mediated glucose uptake. Thus, the present data identify specific sites for therapeutic intervention in the treatment of conditions associated with insulin resistance.

LIST OF ABBREVIATIONS

$[Ca^{2+}]_i$	free cytoplasmic Ca^{2+} concentration
$[Ca^{2+}]_{mem}$	$[Ca^{2+}]$ near the cell membrane
2-APB	2-aminoethoxydiphenyl borate
2-DG	2-deoxyglucose
2-NBDG	2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose
ATP	adenosine trisphosphate
BSA	bovine serum albumin
CaMKII	Ca^{2+}-calmodulin-dependent kinase II
CNT	carbon nanotube
DAG	diacylglycerol
DHPR	dihydropyridine receptor
DMEM	Dulbecco's Modified Eagles Medium
EDL	extensor digitorum longus
EGTA	ethylene glycole tetraacetic acid
ERK	extracellular signal-regulated kinase
FDB	flexor digitorum brevis
FRET	fluorescence resonance energy transfer
GLUT	glucose transporter
$I_{insulin}$	insulin-induced current
I_{OAG}	OAG-induced current
IP_3	inositol 1,4,5-trisphosphate
IR	insulin receptor
IRS	insulin receptor substrates
MAPK	mitogen-activated protein kinase
NMRI	Naval Medical Research Institute
NSCC	non-selective cation current
OAG	1-oleyl-2-acetyl-*sn*-glycerol
ob/ob	obese leptin-deficient
PBS	phosphate buffered saline
PDK1	phosphoinoside dependent kinase 1
PI3K	phosphoinositol 3-kinase
PIP_2	phosphoinositol 4,5-bisphosphate
PIP_3	phosphoinositol 3-phosphate
PKB	protein kinase B
PKC	protein kinase C
PLCγ	phospholipase C isoform γ
PLN	phospholamban
RNS	reactive nitrogen species
ROS	reactive oxygen species
RyR	ryanodine receptor
SERCA	sarcoplasmatic reticulum Ca^{2+}-ATPase
siRNA	small interfering RNA
SR	sarcoplasmatic reticulum
TBP	TATA-box binding protein
TeNT	tetanus toxin
TIRF	total internal reflection fluorescence
TRP	transient receptor potential
TRPC	canonical transient receptor potential
TRPV	vanilloid transient receptor potential
t-SNARE	target SNARE
T-tubule	transverse tubule
v-SNARE	vesicular SNARE
WT	wild type

LIST OF PUBLICATIONS

I Jérémy Fauconnier, **Johanna T Lanner**, Shi-Jin Zhang, Pasi Tavi, Joseph D Bruton, Abram Katz, Håkan Westerblad. *Insulin and inositol 1,4,5-trisphosphate trigger abnormal cytosolic Ca^{2+} transients and reveal mitochondrial Ca^{2+} handling defects in cardiomyocytes of ob/ob mice*. Diabetes 54:2375-2381, **2005**

II Jérémy Fauconnier, **Johanna T Lanner**, Ariane Sultan, Shi-Jin Zhang, Abram Katz, Joseph D Bruton Håkan Westerblad. *Insulin potentiates TRPC3-mediated cation currents in normal but not in insulin-resistant mouse cardiomyocytes.* Cardiovascular Research 73:376-85, **2007**.

III **Johanna T Lanner**, Abram Katz, Pasi Tavi, Marie Sandström, Shi-Jin Zhang, Charlott Wretman, Stephen James, Jérémy Fauconnier, Jan Lännergren, Joseph D Bruton, Håkan Westerblad. *The role of Ca^{2+} influx in insulin-mediated glucose uptake in skeletal muscle*. Diabetes 55:2077-83, **2006**.

IV **Johanna T Lanner**, Joseph D Bruton, Yohannes Assefaw-Redda, Zoita Andronache, Shi-Jin Zhang, Denise Severa, Zhi-Bin Zhang, Werner Melzer, Shi-Li Zhang, Abram Katz, Håkan Westerblad. *Insulin resistance is associated with decreased TRPC3-mediated ion fluxes in adult skeletal muscle cells.* Submitted

Appendix

V **Johanna T Lanner**, Joseph D Bruton, Abram Katz, Håkan Westerblad. *Ca^{2+} and insulin-mediated glucose uptake.* Current Opinion in Pharmacology 8:339-345, **2008**

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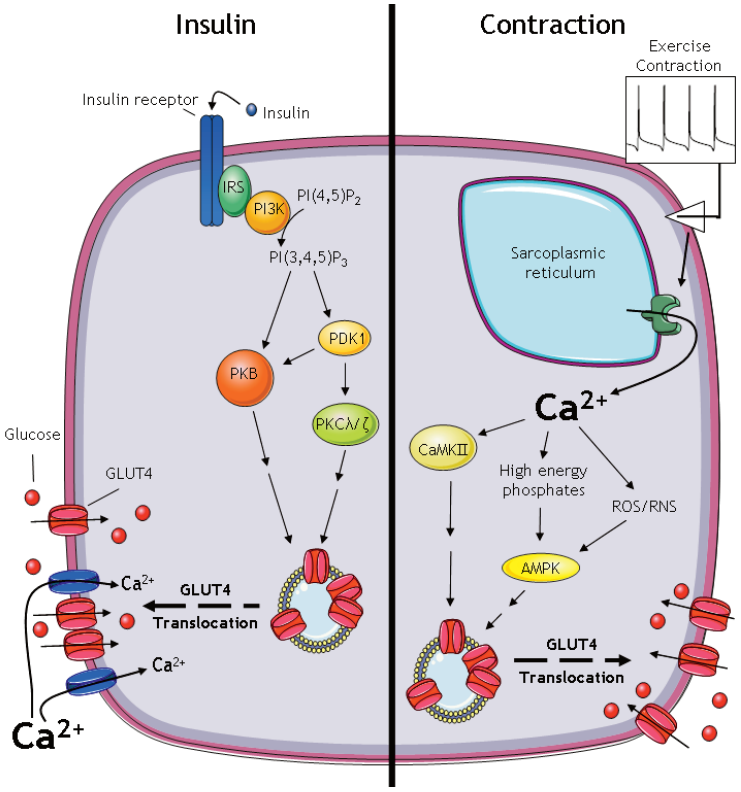
INTRODUCTION

We are currently witnessing a world wide epidemic of people with type 2 diabetes, which is tightly associated with an increased prevalence of obesity and physical inactivity (Wild *et al.*, 2004;Hossain *et al.*, 2007;Cheng, 2005). The estimated number of people with the disease is ~171 million, which correspond to ~35% of the total population of the European Union (Wild *et al.*, 2004)! Even with new therapies, type 2 diabetes is still associated with high morbidity and mortality, and heart disease is the leading cause of death (Gu *et al.*, 1998). As a result, this causes a significant financial burden on the healthcare system (Jonsson, 1998;American Diabetes Association, 2008). Thus, better understanding of the pathological processes in obesity and type 2 diabetes is of major importance, and of particular significance within the context of this thesis.

The progression from insulin resistance to type 2 diabetes

Continuous supply of glucose is essential for proper cell function and in humans the glucose level is maintained within a narrow range close to 5 mM. Altered glucose homeostasis leads to severe complications, including cardiac and renal failure, and damage to the nervous system, all observed in diabetes mellitus (Gu *et al.*, 1998;Jawa *et al.*, 2004;Brownlee, 2005).The term diabetes mellitus describes a metabolic disorder characterized by chronic hyperglycemia with disturbances in carbohydrate, fat and protein metabolism, which result from defects in insulin secretion, insulin action, or both (Alberti & Zimmet, 1998). There are two categories of diabetes; type 1 and 2. Type 1 diabetes (formerly known as insulin-dependent diabetes mellitus) is primarily due to an autoimmune-mediated destruction of pancreatic β-cell islets, usually leading to absolute insulin deficiency (Alberti & Zimmet, 1998;Zimmet *et al.*, 2001). The cause and effect of type 1 diabetes will not be considered further in this thesis. The most common form of diabetes is type 2 diabetes (formerly known as non-insulin-dependent diabetes mellitus), which accounts for ~ 90% of the global cases (Zimmet *et al.*, 2001). Multiple genes together with environmental and behavioral factors, such as sedentary lifestyle and obesity, are expected etiological factors in type 2 diabetes (Zimmet *et al.*, 2001;Cheng, 2005). One major contributor to the progression and pathogenesis of type 2 diabetes is insulin resistance, the cells inability to respond appropriately to normal circulating levels of insulin, resulting in impaired glucose uptake and elevated plasma glucose concentration (DeFronzo, 1997;Shulman, 2000). To sustain insulin

action and physiological blood glucose levels in insulin resistance, the β-cells have to produce and secrete more insulin (DeFronzo, 1997). However, this compensatory process eventually fails, which results in an inability to maintain normal glucose levels (Porte, Jr. & Kahn, 2001;DeFronzo, 1997).



Adapted from Lanner *et al.* Curr Opin Pharmacol, 2008.

Figure. 1. ***Insulin- and contraction-mediated signaling pathways leading to glucose uptake in skeletal muscles and the tentative involvement of Ca^{2+} in these processes.*** Left, following insulin binding and activation of its receptor, a series of signaling events activates translocation of GLUT4 to the surface membrane. At the same time, extracellular Ca^{2+} enters the cell, resulting in localized increases that facilitate the docking/fusion of GLUT4 in the membrane, where it accelerates glucose transport. Right, following membrane depolarization Ca^{2+} is released from SR and contraction starts. This also activates signaling pathways that result in GLUT4 translocation to the surface membrane, where glucose transport is accelerated. Single arrow reflects a direct effect. Multiple arrows in a pathway reflect two or more steps. For the sake of clarity many established steps have been omitted.

Glucose transport over the plasma membrane

The transport of glucose over the plasma membrane is mediated by members of the glucose transporter (GLUT) family and 13 members of this family are known today (Huang & Czech, 2007). Basal glucose uptake occurs via the constitutively active GLUT1, which is expressed in many cell types, including muscle (Mueckler, 1994). GLUT4 is expressed exclusively in striated muscle (i.e. skeletal and cardiac muscle) and adipose tissue (Huang & Czech, 2007;Mueckler, 1994). GLUT4 is stored in intracellular vesicles under basal conditions and upon stimulation these vesicles translocate to the plasma membrane, where GLUT4 inserts in the membrane and glucose uptake is facilitated (Huang & Czech, 2007;Larance *et al.*, 2007;Thong *et al.*, 2005;Bryant *et al.*, 2003).

Skeletal muscle is the major site for insulin-mediated glucose disposal (DeFronzo *et al.*, 1985). Both insulin and exercise (contractions) are able to stimulate GLUT4 recruitment to the plasma membrane in skeletal muscle, although this occurs via different signaling pathways (Thong *et al.*, 2005;Rose & Richter, 2005). In the heart, insulin is the dominant agonist for activating glucose uptake. Continuous heartbeats and strict metabolic control provide a narrow window between basal and contraction-mediated glucose uptake, which makes it difficult to distinguish between the two modes of uptake in cardiac muscle. Moreover, under basal conditions the same argument holds for continuously active slow-twitch skeletal muscles involved in posture, e.g. soleus.

Insulin receptor signaling and GLUT4 translocation in striated muscles

Insulin is involved in a multitude of cellular events including protein synthesis, glucose transport, glycogen synthesis, cell survival, and gene transcription. This thesis concentrates on the branch leading to glucose uptake in striated muscle. The insulin signaling pathway starts with activation of the insulin receptor (IR), which belongs to the tyrosine kinase receptor family. Upon activation, IRs undergo autophosphorylation and recruit and phosphorylate the insulin receptor substrates (IRS) to initiate signal transduction (Thong *et al.*, 2005;White, 2003). IRS activates phosphoinositol 3-kinase (PI3K) resulting in generation of phosphoinositol 3-phosphate (PIP_3), which subsequently activates phosphoinoside dependent kinase 1 (PDK1), Akt (also known as protein kinase B, PKB), and atypical protein kinase C family members (PKCλ/ζ). Eventually this signaling results in translocation of GLUT4-containing vesicles to the plasma membrane and glucose uptake (Bryant *et al.*, 2003;Huang &

Czech, 2007;Thong *et al.*, 2005;White, 2003). At the plasma membrane, activated GLUT4 vesicles undergo docking and fusion through a process that involves a complex of proteins known as SNARE proteins. Documented members of this large SNARE complex are; VAMP-2 (also called synaptobrevin), Munc-18, Synip, and Syntaxin-4 (Ishiki & Klip, 2005;Kanzaki, 2006;Khan *et al.*, 2001;Martin *et al.*, 1996;Olson *et al.*, 1997;Watson & Pessin, 2007;Zaid *et al.*, 2008).The insulin signaling pathway is summarized in Figure 1.

Ca^{2+} signaling in striated muscle

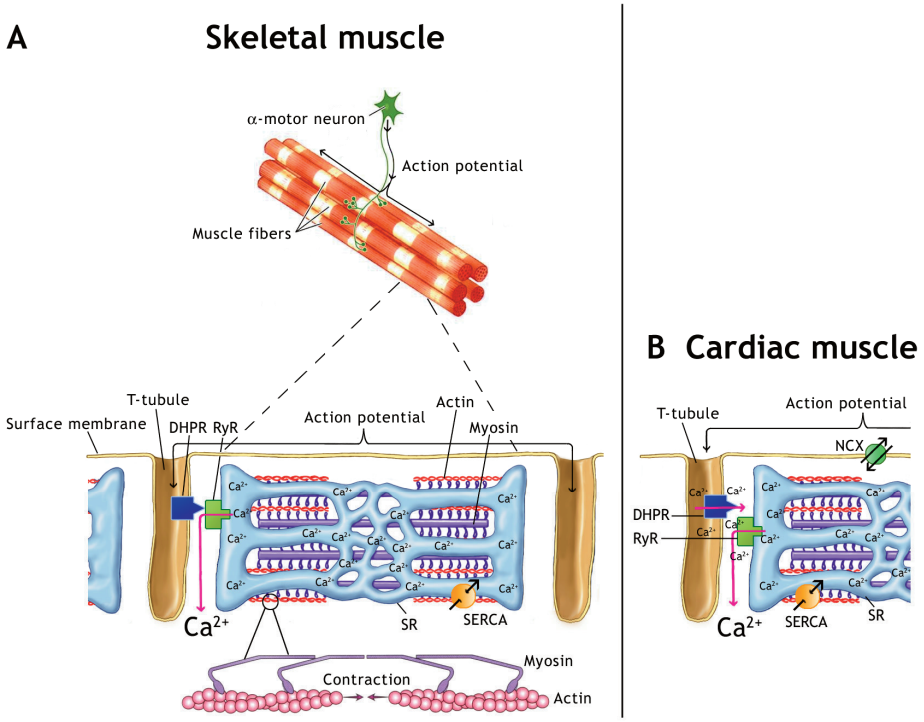
Ca^{2+} and the contractile apparatus

Every heart beat and skeletal muscle contraction is dependent on Ca^{2+}. The activation of the Ca^{2+}-dependent contractile machinery is almost identical in skeletal and cardiac muscle, with exception of the Ca^{2+} release process from the sarcoplasmatic reticulum (SR, equivalent to endoplasmatic reticulum (ER)) (Melzer *et al*., 1995;Bers, 2002).(See figure 2 for schematic picture). In skeletal muscle, action potentials travel along an α-motor neuron to a group of muscle fibers. When the action potential reaches the muscle fibers, the neurotransmitter acetylcholine is released into the neuro-muscular cleft, which results in depolarization of the muscular membrane. This depolarization gives rise to an action potential, which propagates in all direction at the surface membrane and down to the inner parts of the muscle fiber via the transverse tubular (t-tubular) system. The t-tubules are narrow invaginations of the plasma membrane (sarcolemma) located in the A- and I-band interface in mammalian muscle, resulting in a branched network (Dulhunty, 1992;Franzini-Armstrong & Jorgensen, 1994;Franzini-Armstrong, 1991). The action potential in the t-tubules activates voltage sensitive dihydropyridine receptors (DHPRs), also known as L-type Ca^{2+} channels, located in the t-tubular wall. The DHPR are physically in close contact with the ryanodine receptor (RyR) located in the membranes of the SR. When activated, DHPR opens RyR by mechanical interaction. The free cytoplasmic (myoplasmic) Ca^{2+} concentration $[Ca^{2+}]_i$ is very low (~50 mM) under resting conditions, whereas $[Ca^{2+}]$ inside SR is high (~1mM). The low $[Ca^{2+}]_i$ is maintained mainly by the adenosine trisphosphate (ATP) consuming SR Ca^{2+}-ATPase (SERCA) (Carafoli, 1987;Maclennan *et al*., 2002). Following the action potential, opening of RyR results in release of Ca^{2+} from SR and a transient increase in $[Ca^{2+}]_i$. The myoplasmic Ca^{2+} binds to troponin C, initiating movement of tropomyosin, which allows actin and myosin interaction, and force development. After the action potential the SR Ca^{2+} release terminates.

SERCA pumps Ca^{2+} back into SR and $[Ca^{2+}]_i$ returns to resting levels and that subsequently leads to interrupted myosin actin interaction (Dulhunty, 1992;Melzer *et al*., 1995;Stephenson *et al*., 1998). In cardiac muscle, action potentials are initiated by pace maker cells. The action potential is slower (~200 ms) compared to skeletal muscle (~3 ms) (Bers, 2002;Campbell, 1983). Consequently, the DHPRs are open for a sufficient duration to allow for Ca^{2+} influx to occur. Subsequently, these Ca^{2+} ions induce opening of the RyR and Ca^{2+} release from SR, a process known as Ca^{2+}-induced Ca^{2+} release. The increase in $[Ca^{2+}]_i$ enables actin and myosin interaction (as described for skeletal muscle), which eventually results in contraction. In cardiac muscle, $[Ca^{2+}]_i$ returns to resting levels by SERCA-mediated pumping of Ca^{2+} to SR and by extrusion of Ca^{2+} via the Na^{+}-Ca^{2+} exchanger (NCX) (Bers, 2002).

Ca^{2+} dynamics

Ca^{2+} is a multifaceted messenger and apart from enabling activation of contraction it also regulates other cellular events such as fertilization, synaptic transmission, gene transcription, hormonal signaling, metabolism, and cell death. Duration, amplitude, and spatial distribution of Ca^{2+} play a significant role in facilitating these diverse effects of Ca^{2+} (Rizzuto & Pozzan, 2006;Sudhof, 2002;Yoshihara *et al.*, 2003;Berridge *et al.*, 2003). The Ca^{2+}-signaling time scale spans from milliseconds, e.g. in exocytosis of neurotransmitters and in skeletal muscle contractions (Rizzuto & Pozzan, 2006;Sudhof, 2002;Yoshihara *et al.*, 2003;Bers, 2002;Bootman *et al.*, 2006;Lännergren & Westerblad, 1991), up to minutes and hours as observed in fertilization and immune responses (Ozil & Swann, 1995;Lewis, 2001;Dolmetsch *et al.*, 1998). Furthermore, $[Ca^{2+}]_i$ can vary markedly in various cellular loci depending on the conditions (i.e. microdomains of concentration gradients). These gradients can change with time and the alterations can have different effects on various cellular events. For example, local hot spots at the mouth of Ca^{2+} channels cause neurotransmitter release and global prolonged oscillations can affect fertilization of oocytes and gene transcription (Dolmetsch *et al.*, 1998;Rizzuto & Pozzan, 2006;Berridge *et al.*, 2003;Ozil & Swann, 1995;Bootman *et al.*, 2001). Ca^{2+} ions are either derived from intracellular stores by opening of Ca^{2+} release channels, or the extracellular medium via Ca^{2+} influx channels sitting in the plasma membrane (Rizzuto & Pozzan, 2006;Berridge *et al.*, 2003;Putney, Jr., 2007;Pedersen *et al.*, 2005). The work in this thesis focuses mainly on Ca^{2+} influx and canonical transient receptor potential (TRPC) channels (*Papers II-IV*).



Gunilla Danielsson and Johanna Lanner, 2008.

Figure 2. ***Activation of the contractile machinery in skeletal and cardiac muscles.*** **A.** An action potential travels along an α-motor neuron to a group of skeletal muscle fibers. Acetylcholine is released at the neuro-muscular junctions and this triggers an action potential in each of the muscle fibers. The action potential propagates in all directions at the surface membrane and down the t-tubular system where it activates DHPR. The DHPR opens RyR by mechanical interaction resulting in release of Ca^{2+} from SR and a transient increase in $[Ca^{2+}]_i$, which enables actin and myosin interaction, and force development. SERCA pumps Ca^{2+} back into SR and $[Ca^{2+}]_i$ returns to resting levels and the contraction ceases. **B.** The activation of the Ca^{2+}-dependent contractile machinery is almost identical in cardiac muscle, with exception of the Ca^{2+} release process. The action potential is initiated by pace maker cells and propagates along the surface membrane and down the t-tubular system. The cardiac action potentials are slower compared to skeletal muscle, which results in Ca^{2+} influx through DHPRs and these Ca^{2+} ions induce opening of the RyR and Ca^{2+} release from SR. The increase in $[Ca^{2+}]_i$ enables actin and myosin interaction. $[Ca^{2+}]_i$ return to resting levels by SERCA-mediated pumping of Ca^{2+} into SR and by extrusion of Ca^{2+} via NCX.

TRPC channels

The TRPC channels are a subfamily of seven ion channels (designated 1-7) that belong to the large TRP superfamily. The TRP superfamily consists of ~ 30 channels with divergent characteristics, which can be divided into seven main subfamilies based on sequence homology: TRPC, TRPA (ankryin), TRPM (melastatin), TRPML (mucolipin), TRPN (NOMPC), TRPP (polycystin), and TRPV (vanilloid) (Pedersen *et al.*, 2005;Venkatachalam & Montell, 2007). TRPC channels are all Ca^{2+} permeable non-selective cation channels. Application of diacylglycerol (DAG) is known to increase the activity of TRPC3, -6, and 7 in a PKC independent manner (Hofmann *et al.*, 1999;Trebak *et al.*, 2003a;Venkatachalam *et al.*, 2003;Vazquez *et al.*, 2003). Therefore, in *papers II-IV* we used the membrane permeable non-metabolizable DAG analogue, 1-oleyl-2-acetyl-*sn*-glycerol (OAG), as a tool to activate these channels and subsequently induce Ca^{2+} influx. In addition, insulin has been associated with phospholipase C isoform γ (PLCγ) activity and increased DAG levels (Kayali *et al.*, 1998;Eichhorn *et al.*, 2001;Romero *et al.*, 1988;Farese *et al.*, 1988).

The diabetic heart is associated with impaired Ca^{2+} handling

Type 2 diabetes related cardiomyopathy is identified as altered cardiac function independent of conventional cardiac risk factors such as hypertension and atherosclerosis (Fang *et al.*, 2004). Several pathological aspects of type 2 diabetes are characterized by impaired Ca^{2+} handling (Levy, 1999) and hence diabetic cardiomyopathy is known to be associated with altered Ca^{2+} signaling (Levy, 1999;Fang *et al.*, 2004;Chattou *et al.*, 1999;Belke *et al.*, 2004;Pereira *et al.*, 2006). For example, both clinical and experimental studies have shown decreased expression of Ca^{2+} handling proteins and SR dysfunction causing smaller and slower cytoplasmic Ca^{2+} transients (Fang *et al.*, 2004;Chattou *et al.*, 1999;Belke *et al.*, 2004;Pereira *et al.*, 2006).

Ca^{2+} and glucose uptake

Ca^{2+} and contraction-mediated glucose uptake

Increased Ca^{2+} is established as a key component in contraction-mediated glucose uptake in skeletal muscle (Rose & Richter, 2005;Holloszy & Narahara, 1967), see Figure 1. However, the Ca^{2+}-dependent steps are not fully recognized, in part by the difficulty to clearly dissociate between the increases in $[Ca^{2+}]_i$ *per se* and the consequent contraction and contraction-

induced metabolic events. Elevated $[Ca^{2+}]_i$ is known to activate Ca^{2+}-calmodulin-dependent kinase II (CaMKII), which appears to act as an intermediate player in the GLUT4 translocation leading to glucose uptake (Rose & Richter, 2005;Witczak *et al.*, 2007;Wright *et al.*, 2004;Jensen *et al.*, 2007). However, it is possible that increased $[Ca^{2+}]_i$ during contraction can also activate glucose transport by pathway(s) independent of CaMKII (Wright *et al.*, 2004). This pathway(s) appears to be activated under conditions of increased ATP consumption, which results in changes in the concentrations of high energy phosphates and possibly increased levels of reactive oxygen and nitrogen species (ROS/RNS), all of which have been shown to stimulate GLUT4-mediated glucose uptake (Holloszy, 2005;Jessen & Goodyear, 2005;Katz, 2007;Sandström *et al.*, 2006).

Insulin, Ca^{2+} and targeted exocytosis of GLUT4

In contrast to contraction-mediated glucose uptake, the role of Ca^{2+} in insulin-mediated glucose uptake has been debated. There have been several arguments against a role for Ca^{2+} in insulin-mediated glucose transport. For example, incubating skeletal muscles in a solution without added Ca^{2+} did not result in decreased insulin-mediated glucose uptake (Wallberg-Henriksson *et al.*, 1988). Furthermore, insulin did not increase global $[Ca^{2+}]_i$ in various tissues (Klip *et al.*, 1984;Klip & Ramlal, 1987;Cheung *et al.*, 1987). However, earlier research suggested a role for Ca^{2+} in insulin-mediated activation of glucose transport in muscle (Schudt *et al.*, 1976;Bihler *et al.*, 1986;Clausen, 1980). More recently, and in line with previous findings, no effect of insulin was observed on the global free $[Ca^{2+}]_i$ in isolated, intact rodent skeletal muscle fibers (Bruton *et al.*, 1999). However, application of insulin resulted in an increase in $[Ca^{2+}]$ near the cell membrane ($[Ca^{2+}]_{mem}$) in skeletal muscle fibers (Bruton *et al.*, 1999). The increase in $[Ca^{2+}]_{mem}$ appears to depend on Ca^{2+} influx because it was inhibited when extracellular $[Ca^{2+}]$ was decreased to the low nM range (Bruton *et al.*, 1999).

The process of neurotransmitter release is generally induced by a transient and spatially restricted increase in $[Ca^{2+}]_i$ (Sudhof, 2002;Yoshihara *et al.*, 2003;Rizzuto & Pozzan, 2006). Members of the synaptotagmin family are recognized as Ca^{2+} sensors in exocytosis and synaptotagmin I is recognized as the major Ca^{2+} sensor facilitating the vesicle docking and fusion in neurons (Sudhof, 2002;Yoshihara *et al.*, 2003;Rizzuto & Pozzan, 2006). The targeted exocytosis of GLUT4 stimulated by insulin demonstrates many common features with neurotransmitter release, although no Ca^{2+} sensor has yet been explicitly identified (Khan *et al.*, 2001;Martin *et al.*, 1996;Olson *et al.*, 1997). However, a recent report

demonstrates that synaptotagmin VII-deficient mice show altered GLUT4 trafficking and decreased insulin-mediated glucose uptake in adipocytes, but not in skeletal muscle (Li *et al.*, 2007). In adipocytes, hence synaptotagmin VII might function as a Ca^{2+} sensor in insulin signaling. Moreover, Ca^{2+}-calmodulin regulated actin-filament-attached motor protein myosin Myo1c has been shown to associate with and transport GLUT4 vesicles (Bose *et al.*, 2002;Bose *et al.*, 2004;Chen *et al.*, 2007;Huang *et al.*, 2005). Thus, Myo1c is another tentative Ca^{2+} sensitive step in the insulin signaling cascade. In addition, chelation of intracellular Ca^{2+} with BAPTA-AM and application of calmodulin antagonists have been shown to inhibit insulin-stimulated GLUT4 translocation and glucose uptake (Inoue *et al.*, 1999;Whitehead *et al.*, 2004;Shashkin *et al.*, 1995;Yang *et al.*, 2000).

AIMS

The overall aim of this thesis is to investigate the interplay between insulin action and Ca^{2+} fluxes in adult skeletal and cardiac muscle and examine whether the association is altered in obesity and insulin resistance. More specifically, our goals were to:

- Characterize insulin effects on Ca^{2+} homeostasis in ventricular cardiomyocytes of normal and obese, insulin resistant *ob/ob* mice.

- Investigate the role of Ca^{2+} influx in insulin-mediated glucose uptake in skeletal muscle of normal and *ob/ob* mice.

- Examine the interaction between insulin, DAG and TRPC3 channel activity.

EXPERIMENTAL PROCEDURES

For more detailed descriptions see individual papers.

Animals

All animals were housed at room temperature with a 12 h: 12 h light-dark cycle. Food and water were provided *ad libitum*. The following male rodents were used: C57BL/6 mice (*papers I-IV*), NMRI (Naval Medical Research Institute) mice (*paper III*), and obese leptin-deficient *ob/ob* mice with matching wildtype (WT) littermates (*papers I-IV*). Mice were killed by cervical disarticulation and muscles were removed. Male Wistar rats were used as a control in *paper III*. All procedures were approved by the Stockholm North local ethics committee.

Experimental models

Obese ob/ob mice. Leptin is a hormone produced by adipose tissue that regulates energy intake and expenditure (Zhang *et al.*, 1994;Halaas *et al.*, 1995;Campfield *et al.*, 1995). The *ob/ob* mice cannot produce leptin, which results in marked obesity (Zhang *et al.*, 1994). Additional characteristics are increased food intake, insulin resistance, hyperglycemia and hyperinsulinemia (Bray & York, 1979;Coleman, 1978). Thus, *ob/ob* mice exhibit many features in common with the metabolic syndrome and are therefore used as an experimental model in our experiments.

Adult muscle cells. Cellular Ca^{2+} handling is considerably different in adult (mature) skeletal muscle cells as compared to immature muscle cells or cell lines. For instance, $[Ca^{2+}]_i$ transients in response to electrical stimulation have a half-duration of ~5 ms in adult mouse skeletal muscle cells (Baylor & Hollingworth, 2003), which is about 50 times faster than the value obtained in myotubes (~250 ms) (Kubis *et al.*, 2003). Adult cardiomyocytes from intact hearts, in comparison to cell lines, maintain the unique rod-shaped morphology and possesses proteins necessary for contraction, which are features no available cell lines holds (Sambrano *et al.*, 2002). Furthermore, since the adult cardiomyocytes were freshly isolated the t-tubular system was preserved, which is favorable when trying to assess Ca^{2+} signaling that mimics the *in vivo* situation. Consequently, all our experiments were performed on adult cells from skeletal and cardiac muscles.

Cell isolation

Freshly isolated cardiomyocytes *(Papers I and II)*

Single adult ventricular cardiomyocytes were isolated from intact heart following the protocols developed by Alliance for Cellular Signaling (AfCS) (ID PP00000125) (Sambrano *et al.*, 2002). In general, one mouse was killed at ~9 a.m. in the morning of each experimental day. The heart was excised by cutting the aorta about 2-mm from its attachment to the heart, and then the aorta was lifted onto and tied to a cannula. For the heart to remain viable total time to cannulate the heart should be less then 1 min. The cannula was connected to a perfusion system and perfusion of the heart started immediately. The Ca^{2+} free perfusion buffer flushes blood from the vasculature and removes extracellular Ca^{2+} to stop contraction, followed by a buffer containing digestive enzymes (Liberase blendzyme 1, Roche and Trypsin, Gibco) that separates the cardiomyocytes. If the heart is well perfused during the enzyme digestion it swells and turns slightly pale and separation of cardiomyocytes on the surface of the heart may become apparent. After completed enzyme digestion of the heart (~10 min perfusion), the heart was cut from the cannula, and then cut in half to remove the atria from the ventricles. The ventricles were gently teased with forceps into small pieces and then with a pipette to force the cells apart to single cells. Then $Ca^{2+}$was reintroduced and the freshly isolated cardiomyocytes were allowed to rest for at least one hour. Finally, cells were plated on laminin-coated dishes and used for measurements.

Isolation of skeletal muscles *(Papers III and IV)*

The following skeletal muscle preparations were used in *papers III and IV*: whole, isolated extensor digitorum longus (EDL, mainly fast-twitch fibers), whole, isolated soleus (mainly slow-twitch fibers); intact single fibers of flexor digitorum brevis (FDB, mainly fast-twitch fibers) obtained by either enzymatic digestion or by microdissection, and enzymatically dissociated interosseous fibers (mainly fast-twitch fibers) (Liu *et al.*, 1997;Lännergren & Westerblad, 1987).

Enzymatic dissociation (Papers III and IV). Enzymatic dissociation was performed as described by Liu *et al*. 1997 with slight modifications (Liu *et al.*, 1997). Briefly, whole FDB muscles were isolated and placed into a petri dish containing Dulbecco's Modified Eagles Medium (DMEM) with 10% fetal bovine serum (Gibco) and 0.3% collagenase Type I

(Sigma). Muscles were then placed in a water-saturated incubator at 37ºC with 5% CO_2-95% air for ~2 hours. Muscles were then transferred to a dish with fresh DMEM and gently pipetted up and down about 10 times. Dissociated fibers were then transferred to laminin coated plates and allowed to attach for 5-10 minutes before DMEM supplemented with 0.55 µg/ml transferrin (Sigma), 0.50 ng/ml selenium (Sigma), and 1µl/ml antibiotic/antimyotic solution (Sigma). Fibers were then incubated at 37ºC with 5% CO_2-95% air until further use (maximum 48 hours). The interosseous fibers used for two-electrode voltage clamp in *paper IV* were enzymatically dissociated according to the same protocol as above, except that after dissociation the isolated fibers were kept at 4 ºC in a Krebs-Ringer solution with pH 7.6 containing (mM): 118 NaCl, 3.4 KCl, 0.8 $MgSO_4$, 1.2 KH_2PO_4, 11.1 glucose, 25 $NaHCO_3$, and 2.5 $CaCl_2$.

Single fiber microdissection (Paper III). Single fibers were isolated under a dark-field illumination microscope using a pair of jeweller's forceps and micro-iris scissors. The FDB muscles were placed in a chamber containing Tyrode solution consisting of (mM): 121 NaCl, 5.0 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 0.4 NaH_2PO_4, 24 $NaHCO_3$, 0.1 EDTA, and 5.5 glucose. Thereafter, split into three bundles, and then trimmed down to two or three fibers. One fiber was selected and the rest were cut through the sarcolemma, leaving only the selected fiber viable. The fiber's ability to contract was tested by electrical stimulation. After isolation of a single fiber, the tendons were gripped by platinum-foil micro-clips and the preparation was transferred to the perfusion channel of an experimental chamber, placed on the stage of an inverted microscope. The bottom of the chamber consisted of a thin glass cover slip and fibers were mounted as close as possible to this. The fiber was flanked by platinum electrodes that were used for stimulation (Lännergren & Westerblad, 1987).

Ca^{2+} measurements

Cytosolic free Ca^{2+} (Paper I, III and IV)

$[Ca^{2+}]_i$ in *paper I* was measured with the fluorescent Ca^{2+} indicator fluo-3 (20 µM). Changes in fluo-3 fluorescence were measured with confocal microscopy using a BioRad MRC 1024 confocal unit with a krypton/argon mixed gas laser (BioRad Microscopy Division, Hertfordshire, England) attached to a Nikon Diaphot 200 inverted microscope. This confocal microscope was used in all studies. Excitation wavelength was set to 488 nm, and the emitted light was collected through a 522 nm narrow band filter. Experiments were performed in the

line scan mode and scanning was performed along the long axis of the cell. To enable comparisons between the cardiomyocytes, changes in the fluorescence signal (ΔF) were divided by the fluorescence immediately before the stimulation pulse at 1 Hz stimulation (F_o). The time course of Ca^{2+} transients was assessed by line scan setting measuring the time to peak (TTP); the half-width (D½, i.e., the duration at 50% of ΔF); and the time constant (τ) of the exponential part of the decay phase.

The fluorescent Ca^{2+}-indicator indo-1 was used in *paper III and IV* to measure $[Ca^{2+}]_i$ in isolated FDB fibers. Fibers were loaded with the fluorescent indo-1 by incubating them in indo-1-AM (8.3 μM) for 20 minutes and washing for a further 20 minutes. Indo-1 was excited with light at 360 nm and the light emitted at 405 ± 5 and 495 ± 5 nm was measured with two photomultiplier tubes. The ratio (R) of the light emitted at 405 nm to that at 495 nm was translated to $[Ca^{2+}]_i$ as described previously (Andrade *et al.*, 1998).
In *papers I-IV* we only used isolated cardiomyocytes or FDB fibers that gave a robust contraction in response to electrical stimulation at the end of the experiment.

Mitochondrial Ca^{2+} *(Paper I)*
Rhod-2 was used to measure mitochondrial Ca^{2+} as previously described by Trollinger *et al*. 1997 with minor modifications (Trollinger *et al.*, 1997). Briefly, isolated cardiomyocytes were loaded with rhod-2 AM (10 μM) for 30 min at 4°C followed by washout for at least 1 hour rest at room temperature. During the last 30 min of this rest period, cells were also loaded with fluo-3 AM (10 μM) for 20 min followed by 10 min washout. Cells were continuously stimulated at 1 Hz, ensuring stable mitochondrial Ca^{2+} transients. Fluo-3 and rhod-2 fluorescence signals were obtained in the same line scan by excitation at 488 and 568 and measuring emitted light at 522 and 585 nm, respectively. The fluo-3 signal was then used to subtract the cytosolic component of the rhod-2 signal leaving only the mitochondrial component.

Ca^{2+} influx *(Papers III and IV)*
Two highly sensitive methods to assess Ca^{2+} influx were used; Mn^{2+} quenching (Hopf *et al.*, 1996) and Ba^{2+} influx (Trebak *et al.*, 2002). In Mn^{2+} quenching experiments, single FDB fibers were isolated by microdissection and mounted in a stimulation chamber continuously superfused with standard Tyrode solution (no glucose) bubbled with 95% O_2/5% CO_2 to maintain a pH of 7.4. The fiber was microinjected with the fluorescent Ca^{2+} indicator fura-2

and then excited at 360 ± 5 nm (the isosbestic wavelength of fura-2 under our experimental conditions) while measuring the fluorescence light emitted at 495 ± 5 nm. $MnCl_2$ (100 μM) was first added to the bath solution to get the rate of basal quenching. Then a supramaximal concentration of insulin (120 nM = 20 mU/ml). The rate of Mn^{2+} quenching was measured during periods where the rate of decrease of the fluorescent light was stable, i.e. ignoring transient changes induced by changes of the bath solution.

For Ba^{2+} influx experiments, enzymatically isolated FDB fibers were loaded with AM-forms of calcium green-1 (10 μM) or fluo-3 (10 μM). For fluorescence imaging confocal microscopy was used. The cells were excited at 488 nm and the emitted light was collected through a 522 nm narrow band filter. To measure the baseline fluorescence, cells were first superfused with normal Tyrode solution for 5 min. Cells were then exposed to the Ba^{2+} solution, where 1 mM Ca^{2+} was replaced by 1 mM Ba^{2+}, and images were collected at 1 min intervals for a further 10 – 20 min. Data were normalized to the calcium green-1 or fluo-3 fluorescence at the onset of Ba^{2+} exposure, which was set to 1. When used, insulin (120 nM), OAG (30 μM), and the Ca^{2+} influx inhibitor 2-aminoethoxydiphenyl borate (2-APB,100 μM) were added to the Ba^{2+} solution.

Glucose uptake

Glucose uptake in isolated whole muscles *(Paper III)*

Glucose uptake in isolated whole muscles was measured as described by Shashkin *et al*. 1995. Briefly, muscles were placed in vials containing Tyrode buffer with 2 mM pyruvate (no glucose) ± pharmacological agents, e.g. OAG or 2-APB. See individual papers for specific treatments and incubation times of the muscles. The vials were placed in a temperature controlled shaking bath (100 oscillations/min). Radiolabelled 2-deoxy-D-(1,2-^{3}H) glucose (DG) (1mM; 1 mCi/mmol) and inulin (0.2 μCi/ml medium) were used in all experiments and added 20 min prior to freezing. When used, insulin (120 nM) was applied 30 min before addition of 2-DG and inulin. Following incubation, muscles were blotted and frozen in liquid nitrogen. Muscles were then weighed, digested in NaOH at 70°C for 5 min, cooled and centrifuged at 23,000 g for 5 min. Aliquots of the supernatants were added to scintillation cocktail and counted for ^{3}H (2-DG) and ^{14}C (inulin).

Glucose uptake in single FDB fibers *(Paper III and IV)*

Glucose uptake was assessed in enzymatically isolated intact fibers using fluorescent glucose, 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG, 50 μM). Cells were excited at 488 nm while measuring the emitted light at >515 nm. Three sets of fluorescent images were obtained with confocal microscopy. The first image was obtained before application of 2-NBDG (F_a). Fibers were then exposed to 2-NBDG for 15 min and a second image was obtained after 20 min washing (F_b). Fibers were then again exposed to 2-NBDG for 15 min followed by 20 min of washing and the third image was taken (F_c). When used, insulin (60 nM), was present during the last 15 min of the first washing period and during the second exposure to 2-NBDG. For each muscle fiber, the increase in fluorescence during the second 2-NBDG exposure period (ΔF_{c-b}) was divided by the increase in fluorescence during the first exposure (ΔF_{b-a}).

Transfection of adult skeletal muscle fibers

Carbon nanotubes coupled to siRNA targeted against TRPC3 *(Paper IV).*

Carbon nanotubes (CNTs) were used to transfect isolated muscle fibers. Single walled CNTs were prepared as described earlier (Kam *et al.*, 2005) with minor modifications. HiPco® CNTs (Unidym) were sonicated for 90 min in a 0.6% solution of 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)2000] (Avanti Polar Lipids). The suspension was then filtered through a 0.1 μm Millipore filter and re-suspended in a 50 mM phosphate salt buffer. This was centrifuged at 25,000 g for 5 hours to sediment large nanotube bundles. The supernatant was collected and centrifuged again at 25,000 g for a further 5 hours. CNTs were incubated for 1 hour with 2.5 mg sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-propionamido)hexanoate (Pierce) to put reactive sulfhydryl cross-linkers on the CNT surface. Thereafter, small interfering RNA (siRNA), with an extra thiol group attached to the 3' end was added to the CNT and left overnight to allow chemical conjugation to occur.

Intact, adult FDB fibers were isolated and transfected with siRNA by adding 5 μl of the conjugated CNT-siRNA to Petri dishes containing 3 ml DMEM. In control experiments, 5 μl of CNT were added to dishes containing 3 ml DMEM. It has previously been shown that once the CNT enters the cell, the disulfide bond is broken making the siRNA freely available (Kam *et al.*, 2005). The siRNA targeting the TRPC3 was a double strand with sense sequence 5'-UGUCAACUGCGUGGACUAC-3' and antisense sequence 5'-

GUAGUCCACGCAGUUGACA-3'. The specificity of the siRNA sequences was checked against all known mammalian nucleotide sequences using a BLAST search. A randomly scrambled siRNA (nonsense) with sense sequence 5'- AUUCGAGUCCACGUCAGGA-3' and antisense sequence 5'- UCCUGACGUGGACUCGAAU-3' was used to check for specificity of TRPC3 siRNA. siRNAs were labeled with a fluorescent tag (Cy3 or Cy5) attached to the 5' end to check that the constructs entered the cells.

Efficacy of uptake of the conjugated CNT-siRNA was checked by measuring the Cy3 or Cy5 fluorescence inside the cells 48 hours post transfection (Figure 2B, *paper IV*). Western blots were used to determine the levels of TRPC3 protein expression 48 hours post transfection with or without CNT-siRNA targeted against TRPC3 (Figure 2C, *paper IV*). The intensity of the immunoreactive band, at the expected molecular weight (http://www.signaling-gateway.org/molecule), was measured and normalized to the DHPR that was used as a loading control. The TRPC3 protein in the siRNA treated fibers revealed an ~40% decrease compared to control fibers (Figure 2C lower part, *paper IV*; $P < 0.05$). Similar results were obtained when siRNA treated fibers were compared to two other control conditions, i.e. fibers transfected only with CNTs or fibers transfected with nonsense-siRNA (data not shown).

Protein expression, phosphorylation, and interaction

Western blot *(Papers I-IV)*

Western blot was used to measure protein expression. In brief, the protein content of the crude lysate was determined using the Bradford assay (BioRad). Equal amounts of total protein were separated by electrophoresis using NuPAGE Novex Gels (Invitrogen) and transferred onto a polyvinylidine fluoride (PVDF) membrane (BioRad). Membranes were blocked in 5% (w/v) non-fat milk (or 5% bovine serum albumin (BSA) (w/v)) in Tris-buffered saline containing 0.05% Tween 20 followed by incubation with primary antibodies. Membranes were then incubated with secondary horseradish peroxidase-conjugated antibody and immunoreactive bands were visualized using enhanced chemiluminescence (Super Signal, Pierce). Western blot analysis was performed using the following antibodies: rabbit anti-TRPC3, and -TRPC4 (Alomone Labs), mouse anti-DHPR (Abcam), rabbit anti-GLUT4 (Chemicon), mouse anti-GLUT4 (Acris), phosphorylated and total extracellular signal-regulated kinases (ERK1/2) (also known as classical mitogen-activated protein kinases (MAPK), New England Biolabs)).

Elisa *(Papers III and IV)*

Insulin-mediated phosphorylation of Akt was measured with Elisa. Isolated FDB fibers and whole EDL muscles were incubated at 35ºC ± 60 nM and 120 nM insulin, respectively. Muscles and fibers were then frozen in liquid nitrogen. Preparation and measurement of phoshorylated Akt and total Akt were performed using PathScan Elisa Kit (Cell Signaling #7160 and #7170, respectively) following the supplier's protocol. In initial experiments, the protein concentrations that fell in the linear range were established and subsequently used in the analyses.

Immunoprecipitation *(Papers II and IV)*

Immunoprecipitation was used to examine protein-protein interaction. Equal amounts of protein were incubated with primary antibody overnight at 4˚C with rotation. Then 30 µl protein G agarose suspension (Santa Cruz Biotechnology) were added to the samples followed by 4 hour incubation at 4°C, with rotation. Samples were then washed three times, eluted with 40 µl LDS sample buffer (Invitrogen), and heated for 10 min at 70°C. Western blots were then performed as described above.

Immunofluorescence staining *(Papers II and IV)*

Freshly dissociated cardiomyocytes (*paper II*) and FDB fibers (*paper IV*) were placed on laminin-coated dishes in Tyrode solution. Fibers were fixed in phosphate buffered saline (PBS) with 0.2% paraformaldehyde for 20 min and then permeabilized by 0.3% Triton X-100 in PBS solution. After rinsing, fibers were preincubated for 30 min in 10% normal goat serum. Fibers were then incubated with primary antibody diluted in PBS containing 1% BSA at 4°C overnight. They were then washed and incubated with fluorescent secondary antibody. Images of longitudinal thin sections of stained cells were obtained with laser confocal microscopy. Antibodies used were: rabbit anti-TRPC3 (1:50 dilution, Alomone Labs), mouse anti-GLUT4 (1:25, Acris), mouse anti-DHPR (1:100, Abcam), Texas Red (1:100, Santa Cruz Biotechnology), and Alexa Fluor 488 (5 µg/ml, Molecular Probes/ Invitrogen).

TRPC3 gene expression

RNA isolation and quantitative real time PCR *(Paper IV)*

The mRNA levels of TRPC3 were measured in isolated EDL muscles from WT and *ob/ob* mice with quantitative real time PCR (RT-PCR) analysis. Total RNA from WT and *ob/ob* muscles tissue samples was isolated using TRIZOL Reagent (Gibco). Purified total RNA was DNase treated (DNA-free DNase Treatment & Removal Reagents, Ambion) and used as a template for cDNA synthesis (TaqMan Reverse Transcription Reagens, Applied Biosystems) according to the manufacturer's protocol. Each targeted gene was measured in triplicate using TaqMan Universal PCR reagent mix (Applied Biosystems) with 10 ng target cDNA. Experiments were performed in 96-well optical plates using an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). Primers and fluorogenic probes for mTRPC3 were purchased from Applied Biosystems. PCR amplification parameters were: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The data were normalized to TATA-box binding protein (TBP), which was found to show little difference between muscles.

Measurement of non-selective cation currents

Voltage clamp *(Papers II and IV)*

Voltage clamp was used to measure non-selective cation currents (NSCCs) in freshly isolated cardiomyocytes (*paper II*) and in enzymatically dissociated interosseous fibers (*paper IV*). Whole-cell patch clamp was used for the cardiomyocytes and voltage clamp with a two-electrode technique was used for interosseous fibers. Experiments were performed at room temperature (~22-24 ºC). After isolation, cardiomyocytes were placed in a perfusion chamber and continuously perfused with standard Tyrode solution. Whole-cell patch clamp experiments were performed with an Axopatch 200B (Axon Instruments). Patch pipettes had a resistance of 2-3 MΩ. Currents were normalized to the cell membrane capacitance and presented as current densities (pA/pF). Series resistances were electronically compensated before voltage clamp recordings. Leak current was not compensated and all cells exhibiting a current leak larger than 100 pA were excluded. The patch pipette solution contained (mM): 120 CsCl, 6.8 $MgCl_2$, 5 Na_2ATP, 5 $Na_2creatinePO_4$, 0.4 Na_2GTP, 11 EGTA, 4.7 $CaCl_2$ (120 nM free $[Ca^{2+}]$), and 20 HEPES; pH was adjusted with CsOH to 7.2. To record an NSCC induced by insulin and/or OAG, the bathing solution was switched to a modified Tyrode where: Na^+ was replaced by Li^+ to inhibit the Na^+-Ca^{2+} exchanger; 200 μM ouabain was

added to inhibit Na^+-K^+ ATPase; 1 mM $BaCl_2$ was added to block residual K^+ currents and background conductance. Quasi steady-state I-V relations of the NSCC were obtained by applying a descending voltage ramp. The voltage ramp went from +50 mV to -120 mV with a slope of 0.057 V/s and was repeated at 0.03 Hz. Insulin- or OAG-mediated NSCCs were determined as the difference between steady state currents recorded after and before application of drugs.

For skeletal muscles fibers, isolated interosseus fibers were voltage clamped in a two-microelectrode configuration using an Axoclamp 2B amplifier (Axon Instruments). Experiments were performed in an external solution of the following composition (mM): 125 tetraethylammonium hydroxide, 145 HCH_3SO_3, 2 $MgCl_2$, 10 $Ca(OH)_2$, 5 4-aminopyridine, 10 HEPES, 0.001 Tetrodotoxin, 5 glucose, 0.5 antrachene-9-carboxylic acid, 0.05 *N*-benzyl-*p*-toluene sulphonamide (pH 7.4). The voltage-recording electrodes were filled with 3 M KCl and had a resistance of 6.9 ± 0.4 MΩ (n = 41) when measured in extracellular solution. The current passing patch electrodes were filled with internal solution containing (mM): 145 CsOH, 110 aspartic acid, 0.75 Na_2ATP, 4.25 MgATP, 1.5 $CaCl_2$, 10 HEPES, 15 EGTA, 5 $Na_2creatinePO_4$, 0.4 Na_2GTP (pH 7.2); anti-TRPC3 antibodies (1:25 dilution) were added to the patch pipette solution in some experiments. The patch pipette resistance was 2.5 ± 0.1 MΩ (n = 41). After inserting the voltage-recording electrode, the control voltage was set to -80 mV. Then the current-passing electrode was gently sealed to the membrane and the previously applied positive pressure was released, which usually led to establishing the contact to the cytosol. Thereafter, a 30 min loading period was applied to allow the artificial intracellular solution in the patch pipette to diffuse into the fiber. After the loading period was finished, the holding potential was increased to +10 mV to inactivate L-type Ca^{2+} currents. The descending voltage ramp from +50 mV to -120 mV and drugs was then applied as described for cardiomyocytes.

RESULTS

This section will consist of two separate parts. The first part will present results from *papers I and II* focusing on Ca^{2+} handling in cardiomyocytes. The second part will present results from *papers III and IV*, with focus on the role of Ca^{2+} in insulin-mediated glucose uptake in skeletal muscles.

Papers I and II

Intracellular Ca^{2+} handling is altered in cardiomyocytes of *ob/ob* mice

Heart disease is a leading cause of death in patients with type 2 diabetes (Stamler *et al.*, 1993;Wilson, 2001;Bell, 2003), and impaired intracellular Ca^{2+} handling has been put forth as a fundamental problem in the development of cardiomyopathy in obesity and type 2 diabetes (Belke *et al.*, 2004;Pereira *et al.*, 2006;Chattou *et al.*, 1999). For better understanding of the pathological processes we investigated Ca^{2+} handling in *ob/ob* cardiomyocytes.

Cytoplasmic Ca^{2+} transients are smaller in ob/ob cardiomyocytes

We studied the characteristics of Ca^{2+} transients recorded during 1 Hz stimulation in WT and *ob/ob* mouse ventricular cardiomyocytes. Under control conditions, the amplitude of Ca^{2+} transients was smaller and the decay phase was slower in *ob/ob* as compared to WT cardiomyocytes (Figure 1, *paper I*). Contractions were also markedly weaker in *ob/ob* cells as evidence by their shortening by 7.1 ± 0.8% of their resting length (n = 21) as compared to 15.5 ± 1.8% (n = 33) in WT cells. The results are in accordance with findings from different models of obesity and type 2 diabetes (Pereira *et al.*, 2006;Belke *et al.*, 2004). Their smaller and slower Ca^{2+} transients were associated with decreased SR Ca^{2+} load (Pereira *et al.*, 2006) and increased expression of the SERCA inhibitory protein phospholamban (PLN) (Belke *et al.*, 2004), which partly may explain the contractile dysfunctions obtained here and observed in different models of obesity and type 2 diabetes (Pereira *et al.*, 2006;Belke *et al.*, 2004;Carroll *et al.*, 1997;Ren & Bode, 2000;Christoffersen *et al.*, 2003).

Effects of insulin and IP_3 on cytoplasmic Ca^{2+} transients

Application of insulin (60 nM) significantly increased the Ca^{2+} transient amplitude by ~30% in WT cells, whereas there was no significant effect on the time-course (Table 1, *paper I*). On the other hand, insulin did not produce any significant change of the Ca^{2+} transient amplitude in *ob/ob* cardiomyocytes, but the transient became broader. This broadening of the transient was due to an increased time-to-peak and slowed onset of the decay phase while the rate of decline during the final phase, if anything, tended to be faster in the presence of insulin (Table 1, *paper I*). Application of insulin triggered frequent extra Ca^{2+} transients during the final part of the decay phase in 10 out of 18 *ob/ob* cardiomyocytes (Figure 2, *paper I*). Such extra Ca^{2+} transients were not observed under control conditions in *ob*/*ob* cells, nor were they seen in WT cells even in the presence of insulin.

One central and early event in intracellular insulin signaling is the activation of PI3K (Khan & Pessin, 2002;White, 2003). To determine the involvement of the PI3K pathway, we pretreated cells with the PI3K inhibitor wortmannin (0.5 μM) (Davies *et al.*, 2000), which completely blocked the effects of insulin on Ca^{2+} transients in both WT and *ob*/*ob* cells (Table 1, *paper I*), which suggests that the effects of insulin occurred via the PI3K-dependent signaling pathway.

Several studies have shown that inositol 1,4,5-trisphosphate (IP_3) can induce cardiac arrhythmias (Jacobsen *et al.*, 1996;Zima & Blatter, 2004;Mackenzie *et al.*, 2002). In addition, insulin has been shown to interfere with IP_3 signaling in neonatal cardiomyocytes (Kudoh *et al.*, 2002). We tested the effect of a membrane-permeant IP_3 analogue (2,4,6-tri-O-butyryl-I[1,3,5]P_3, 10μM) on Ca^{2+} transients. Application of the IP_3 analogue had no significant effect on the amplitude of Ca^{2+} transients in *ob/ob* or WT cardiomyocytes (Table 1, *paper I*). However, it produced broader Ca^{2+} transients in both groups due to an increased time-to-peak and slowed early decay phase whereas the rate of the final decay was not affected. Moreover, the IP_3 analogue induced extra Ca^{2+} transients in 8 out of 13 *ob/ob* cells, while only 2 out of 18 WT cells showed such transients (Figure 2B & D, *paper I*).

Mitochondrial Ca^{2+} transients

Mitochondria are known to contribute to the shaping of Ca^{2+} signals in cardiomyocytes (Mackenzie *et al.*, 2004) and diabetes is associated with impaired mitochondrial function (Levy, 1999;Lowell & Shulman, 2005;Duchen, 2004;Brownlee, 2005;Mazumder *et al.*, 2004). Therefore, altered mitochondrial Ca^{2+} uptake in a beat-to-beat manner may have a role in the difference between Ca^{2+} signals in WT and *ob*/*ob* cardiomyocytes. We recorded transients of mitochondrial rhod-2 fluorescence at 1 Hz stimulation in the absence or presence of insulin (60 nM). There was no difference between WT and *ob/ob* cells regarding the amplitude of mitochondrial Ca^{2+} transients under control conditions. However, the transients were significantly ($P < 0.05$) slower in *ob/ob* compared to WT cells, with the time to peak and half-width being 182 ± 19 and 325 ± 24 ms in *ob*/*ob* cells (n = 14) *vs*. 115 ± 21 and 222 ± 20 ms in WT cells (n = 12), respectively. Application of insulin significantly increased the amplitude of mitochondrial Ca^{2+} transients in WT cells, whereas the amplitude was not changed in *ob*/*ob* cells (Figure 5, lower part, *paper I*). Application of the IP_3 analogue also increased the amplitude in WT but not in *ob*/*ob* cells. Thus, the dynamic mitochondrial Ca^{2+} buffering during insulin or IP_3 exposure was blunted in *ob/ob* cardiomyocytes.

Insulin and the IP_3 concentration

Activated PLCγ is known to induce hydrolysis of phosphoinositol 4,5-bisphosphate (PIP_2), which generates increases in total cellular DAG and IP_3. Since insulin has been associated with PLCγ activity (Eichhorn *et al.*, 2001;Kayali *et al.*, 1998), we measured the IP_3 concentration in WT and *ob*/*ob* cardiomyocytes in the absence and presence of insulin. Insulin had no effect on the IP_3 concentration in WT cells whereas it significantly increased the concentration by ~30% in *ob*/*ob* cells ($P < 0.05$; Figure 3D, *paper I*).

Characterization of OAG-induced plasma membrane ion currents

In the next set of experiments (*paper II*), NSCCs were measured in cardiomyocytes, focusing on the interaction between insulin, DAG and TRPC3 channels. Ion fluxes were stimulated or inhibited by pharmacological agents that are frequently used to manipulate Ca^{2+} influx through non-selective cation channels. The membrane permeable non-metabolizable DAG analogue OAG that is known to increase the activity of TRPC3 and stimulate Ca^{2+} influx (Hofmann *et al.*, 1999;Trebak *et al.*, 2003a;Venkatachalam *et al.*, 2003;Vazquez *et al.*, 2003),

the Ca^{2+} influx inhibitors 2-APB (30 μM), Gd^{3+} (1 μM), SK&F96365 (10 μM) (Bootman *et al.*, 2002;Trebak *et al.*, 2002;Lievremont *et al.*, 2005;Putney, Jr., 2001), and the anti-TRPC3 antibody (Albert *et al.*, 2006) were used. In the presence of OAG (30 μM), an outwardly rectifying current developed within minutes in both WT and *ob/ob* cardiomyocytes (Figure 1A, *paper II*). The maximum amplitude of the OAG-induced current (I_{OAG}) was significantly smaller in *ob/ob* (1.3 ± 0.3 pA/pF at +50mV) compared to WT cardiomyocytes (2.2 ± 0.2, Figure 1B, *paper II*). 2-APB, SK&F96365, and Gd^{3+}, significantly reduced I_{OAG} in WT and *ob/ob* cardiomyocytes (Figure 1B, *paper II*). In addition, similar findings were obtained in skeletal muscle fibers (see below, *paper IV*). The primary role of IP_3 is to mobilize Ca^{2+} from intracellular stores and not in the activation of plasma membrane ion currents (Berridge *et al.*, 2003). As expected, application of the IP_3 analogue did not induce NSCC in WT cells and it had no significant effect on the NSCC in the presence of OAG as compared to application of OAG on its own.

Insulin activates an NSCC and can potentiate OAG-induced currents

Insulin has been shown to activate a dose- and time-dependent NSCC ($I_{insulin}$) in adult guinea pig cardiomyocytes (Zhang & Hancox, 2003). Insulin activated an NSCC in a dose- and time-dependent manner also in mouse cardiomyocytes and this current was similar in WT and *ob/ob* cardiomyocytes (Figure 2A & B, *paper II*). The reversal potential (E_{rev}) of $I_{insulin}$ was not different between WT and *ob/ob* cells (E_{rev}= 7.9 ± 2.0 mV for WT (n = 10) vs. 8.6 ± 2.2 mV for *ob/ob* (n = 9)), but it was significantly more positive than E_{rev} for I_{OAG} (~ -9 mV). SK&F96365 (10 μM), Gd^{3+} (1 μM), and wortmannin (0.5 μM) significantly decreased $I_{insulin}$ in both WT and *ob/ob* cells. On the other hand, application of 2-APB (30 μM), did not affect $I_{insulin}$ in either WT or *ob/ob* cells (Figure 2C, *paper II*).

The interaction between insulin and OAG was tested by first exposing cardiomyocytes to insulin (60 nM) for 20 min, which gave a stable $I_{insulin}$, and then adding OAG (30 μM). In WT cardiomyocytes, this resulted in a marked increase in current density; I_{OAG} was significantly larger (~50%) in the presence than in the absence of insulin (Figure 3Ab, *paper II*). In contrast, insulin did not induce an increase in I_{OAG} in *ob/ob* cardiomyocytes (Figure 3Bb, *paper II*). Moreover, the potentiating effect of insulin on I_{OAG} was abolished in the presence of wortmannin. In summary, pre-treatment with insulin resulted in a wortmannin-dependent increase in I_{OAG} in WT but not in *ob/ob* cardiomyocytes.

Tetanus toxin (TeNT) cleaves VAMP2 (Pellizzari *et al.*, 1996;Sutton *et al.*, 1998) and hence prevents the interaction between vesicle (v)- and target (t)-SNARE proteins (Singh *et al.*, 2004). We used TeNT in the patch pipette (10 nM) to investigate whether SNARE proteins are involved in the insulin-mediated potentiating of I_{OAG} cardiomyocytes. In the presence of TeNT, insulin pre-treatment failed to increase I_{OAG} in WT cardiomyocytes (Figure 3Ab, *paper II*).

The I_{OAG} was inhibited by anti-TRPC3 antibody

In arterial smooth muscle cells the anti-TRPC3 antibody used in the present study can block cation currents when applied on the inside of the plasma membrane (Albert *et al.*, 2006). We therefore measured $I_{insulin}$ and I_{OAG} with anti-TRPC3 antibody (+/- an excess of TRPC3 blocking peptide) present in the patch pipette. $I_{insulin}$ was not inhibited by addition of anti-TRPC3 antibodies to the patch pipette solution. On the other hand, the OAG-induced (30 μM) current in the presence of anti-TRPC3 antibody was significantly decreased (at + 50 mV) both in the absence (~50%) and presence of insulin (> 90%, Figure 4, *paper II*). Thus, these results indicate a direct role of TRPC3 in the OAG-induced but not the insulin-induced NSCC.

Modest difference in protein expression levels between WT and ob/ob hearts

IP_3 receptor type 2 is the predominant isoform in cardiomyocytes (Lipp *et al.*, 2000). We used western blot to determine the protein expression and the mean expression of IP_3 receptor type 2 was not different between *ob/ob* and WT ventricles (Figure 3C, *paper I*). However, the TRPC3 expression showed a modest, but significant ($P < 0.05$), decreased expression in *ob/ob* (57 ± 6%) compared to WT (100 ± 5%) ventricles (Figure 5A, *paper II*).

TRPC3 and GLUT4 co-immunoprecipitates

Both GLUT4 and TRPC3 can translocate from intracellular vesicles to the plasma membrane in a VAMP2-dependent manner (Bryant *et al.*, 2003;Foster & Klip, 2000;Singh *et al.*, 2004). We used immunoprecipitation followed by western blot and showed that TRPC3 was detected in GLUT4 immunoprecipitates both in the absence and presence of insulin (60 nM) (Figure 5B, *paper II*). Later, these experiments were repeated in skeletal muscle (*paper IV*) and similar findings were obtained (see Figure 4B, *paper IV*).

Insulin induced a translocation of TRPC3 in WT cardiomyocytes

Immunofluorescence staining was used to study the intracellular localization of TRPC3 in ventricular cardiomyocytes of WT and *ob/ob* mice. In the absence of insulin, TRPC3 staining was observed directly on as well as immediately beneath the cell surface membrane (Figure 6D, *paper II*). Little staining was seen in the centre of the cell and the t-tubular system could not be discerned. Treatment of WT cardiomyocytes for 20 min with insulin (60 nM) concentrated TRPC3 staining to the cell surface membrane and decreased the subsarcolemmal staining; the relative staining at 0% from the cell edge was 0.98 ± 0.02 with insulin and 0.77 ± 0.05 without ($P < 0.05$) and at 5% from the cell edge it was 0.32 ± 0.03 with insulin and 0.75 ± 0.04 without ($P < 0.05$). Moreover, punctuated TRPC3 staining at the subsarcolemmal level was observed under control conditions, whereas the staining was more uniformly distributed along the plasma membrane after insulin treatment (Figure 6D, *paper II*). This suggests that TRPC3 was stored in vesicles in the vicinity of the sarcolemma before insulin application. In addition, application of wortmannin (0.5 μM) completely abrogated insulin-mediated TRPC3 translocation (Figure 6B & 7A, *paper II*). Exposure of WT cardiomyocytes to OAG (30 μM) did not cause any detectable translocation of TRPC3; the relative fluorescence at 0% and 5% from the cell edge was 0.68 ± 0.09 and 0.72 ± 0.07 (n = 9), respectively.

In the absence of insulin, *ob/ob* and WT ventricular cardiomyocytes showed a similar TRPC3 staining pattern (Figure 6C and E, *paper II*). However, insulin treatment failed to cause any redistribution of TRPC3 to the plasma membrane in *ob/ob* cardiomyocytes (Figure 7B, *paper II*).

Papers III and IV

Ca^{2+} influx plays an important role in insulin-mediated glucose uptake

Increased insulin sensitivity and improved glucose homeostasis are two goals in treatment of patients with type 2 diabetes. The involvement of Ca^{2+} in insulin-mediated glucose uptake has been debated. We examined the role of Ca^{2+} in insulin-mediated glucose uptake and propose TRPC3 as a therapeutic target for treatment of insulin resistant conditions.

The effects of 2-APB on insulin- and OAG-induced Ca^{2+} influx

We have previously shown that insulin increases $[Ca^{2+}]$ near the plasma membrane, $[Ca^{2+}]_{mem}$, but has no effect on global $[Ca^{2+}]_i$ (Bruton *et al.*, 1999). These findings were further investigated with measurements of insulin- and OAG-induced Ca^{2+} fluxes using Mn^{2+} quenching of fura-2 fluorescence (Hopf *et al.*, 1996) and Ba^{2+} influx measured with Ca^{2+} indicators (calcium green-1, fluo-3) (Trebak *et al.*, 2002). An attraction with these methods is that they are very sensitive and can detect even small changes in the Ca^{2+} influx.

The basal rate of Mn^{2+} quenching was $0.70 \pm 0.06\%$ min^{-1} ($n = 11$). Insulin increased the rate of quenching in four out of five cells tested (Figure 1B, *paper III*). The subsequent application of 2-APB (100 μM) resulted in a significant reduction of the rate of quenching ($P < 0.01$). Surprisingly, removal of 2-APB in the continued presence of insulin gave a rebound effect with a rate of quenching that was ~30% higher than basal ($P < 0.01$), although other studies have also shown enhanced Ca^{2+} entry in presence of 2-APB (Bootman *et al.*, 2002;Prakriya & Lewis, 2001). Noteworthy, the mechanism behind the activity of 2-APB is unclear, but it is known to inhibit both intracellular Ca^{2+} release and Ca^{2+} influx, although it has been shown to be a more reliable inhibitor of Ca^{2+} influx (Bootman *et al.*, 2002;Iwasaki *et al.*, 2001;Prakriya & Lewis, 2001;Ma *et al.*, 2003;Ma *et al.*, 2002;Ma *et al.*, 2001). The inhibitory action of 2-APB is dependent on e.g. concentration; considerably different concentrations of 2-APB are needed to inhibit IP_3-induced Ca^{2+} release (~nM - μM), whereas Ca^{2+} influx is generally fully inhibited by 50-100 μM 2-APB (Ma *et al.*, 2003;Ma *et al.*, 2002;Missiaen *et al.*, 2001;Bilmen *et al.*, 2002;Mackenzie *et al.*, 2002). Thus, depending on the experimental conditions 2-APB can be used as an inhibitor of both Ca^{2+} release and Ca^{2+} influx. It is possible that when 2-APB affected insulin-mediated changes in $[Ca^{2+}]_i$, this was due to effects on L-type Ca^{2+} channels and/or IP_3-receptors in the skeletal muscle cells (Bootman *et al.*, 2002;Peppiatt *et*

al., 2003). On the other hand, L-type Ca^{2+} channels are normally activated by depolarization and insulin causes hyperpolarization of the sarcolemma (Bruton *et al.*, 1999;Clausen & Flatman, 1987) and IP_3 are mainly important during development and its role in adult skeletal muscle is uncertain (Berchtold *et al.*, 2000). Thus, even if 2-APB inhibited L-type Ca^{2+} channels and IP_3-mediated Ca^{2+} release, neither of these events appear to be associated with an increase in $[Ca^{2+}]_i$ in response to insulin (Wallberg-Henriksson *et al.*, 1988;Klip *et al.*, 1984;Klip & Ramlal, 1987;Bruton *et al.*, 1999).

Application of insulin caused a significant increase in the Ba^{2+} influx (~20 %, $P < 0.001$) and an influx of the same magnitude was observed in the presence of OAG (30 µM) (Figure 1C & D, *paper III*). Application of 2-APB in the presence of insulin or OAG abolished the insulin- or OAG-induced increase in Ba^{2+} influx (Figure 1D, *paper III*).
We also studied the combined effect of insulin plus OAG on Ba^{2+} influx (Figure 1E, *paper III*). Interestingly, application of OAG in the continued presence of insulin, revealed an ~40% additional increase in the signal as compared to the increase induced by insulin alone ($P < 0.001$).

Knock down of TRPC3 inhibits OAG-induced Ca^{2+} influx

One disadvantage with pharmacological tools is the risk of lack of specificity, which limits the possibility to identify the channel/s responsible for the Ca^{2+} influx. To be able to make a comprehensive investigation of the role of TRPC3 in insulin-mediated glucose uptake it became necessary to knock down the protein of interest. However, adult muscles are difficult to transfect. In *paper IV* we developed a new transfection technique with siRNA linked to functionalized CNTs, which were successful and protein expression of TRPC3 was knocked down. Functionalized CNTs exhibit advantages compared to other available transfection methods, which are shown to be more complex to use and less effective (e.g. adenovirus and cationic lipid mixtures such as Lipofectamine) (Kam *et al.*, 2005;Sapru *et al.*, 2002;Pramfalk *et al.*, 2004). Endocytosis is the suggested mechanism for how functionalized CNTs enter cells (Kam *et al.*, 2006;Kam *et al.*, 2004). The CNT-based transfection with siRNA targeted against TRPC3 resulted in an ~40% reduction (Figure 2C, *paper IV*) in protein expression with no signs of unspecific effects or cell damage. Thus, in addition to other currently used transfection methods (i.e. adenovirus and cationic lipid mixtures), functionalized CNT provides a potent and specific tool to introduce siRNA into intact adult skeletal muscle.

OAG-induced Ba^{2+} influx was measured to study the consequences of TRPC3 knock down on channel activity. Application of OAG (30 μM) caused a significant increase in Ba^{2+} influx in control fibers and this increase was reduced by ~70% in TRPC3 knock down fibers (Figure 3C, *paper IV*). Knock down of TRPC3 had no generalized effect on $[Ca^{2+}]_i$ handling, as judged from normal tetanic $[Ca^{2+}]_i$ transients (Figure 3A, *paper IV*). Thus, knock down of TRPC3 protein expression inhibited the OAG-induced Ba^{2+} influx, and by analogy DAG-induced Ca^{2+} influx.

The TRPC3/6/7 proteins have ~75% protein homology and are associated with similar functions (Trebak *et al.*, 2003b). One possibility is then that TRPC6 would take over the role of TRPC3 in TRPC3 knock down fibers (TRPC7 is not expressed fast-twitch in skeletal muscle, data not shown, (Vandebrouck *et al.*, 2002)). Based on the finding that knock down of TRPC3 resulted in a decrease in OAG-mediated Ca^{2+} influx indicates that this does not occur, at least not in the 48 hours time frame of our TRPC3 knock down studies. Because if TRPC6 would take over the role of TRPC3 one would expect that no decrease in Ca^{2+} would be detected due to increased flux via TRPC6.

I_{OAG} are inhibited by anti-TRPC3 antibody and are smaller in ob/ob than in WT skeletal muscle fibers

For a direct measurement of the activity of ion fluxes over the plasma membrane in skeletal muscles voltage clamp was performed on adult muscle fibers. Application of OAG (30 μM) induced a current, I_{OAG}, with an almost linear current-voltage relationship (Figure 1A, *paper IV*). I_{OAG} was almost completely blocked with anti-TRPC3 antibody (1:25 dilution) in the patch pipette, indicating that the current is conducted via TRPC3. I_{OAG} was also inhibited by 2-APB (100 μM) and Gd^{3+} (1 μM) (Figure 1B, *paper IV*). I_{OAG} was significantly smaller (0.64 ± 0.1 pA/pF at + 45mV) in insulin-resistant *ob/ob* compared to WT muscle cells (4.2 ± 0.2 pA/pF at + 45mV), whereas the reversal potential was the same in the two groups (WT, 8.9 ± 1.5 mV; *ob/ob*, 8.9 ± 0.5 mV). The smaller I_{OAG} in *ob/ob* cells was not due to decreased TRPC3 expression, because TRPC3 content was similar in WT and *ob*/*ob* muscles both at the protein (Figure 1C, *paper IV*) and mRNA level (Figure 1D, *paper IV*).Taken together, OAG induced a TRPC3-dependent NSCC in both cardiomyocytes (*paper II*) and skeletal muscle fibers and this current was smaller in *ob/ob* compared to WT cells.

Decreased Ca^{2+} influx is associated with decreased insulin-mediated glucose uptake

Earlier studies showed no decrease in insulin-mediated glucose uptake when muscles were bathed in a solution with no added Ca^{2+} (Wallberg-Henriksson *et al.*, 1988;Henriksen *et al.*, 1989), and such findings may form a basis for not recognizing a role for Ca^{2+} influx in insulin signaling. In accordance with previous studies on isolated whole muscles (Wallberg-Henriksson *et al.*, 1988;Henriksen *et al.*, 1989), we did not detect any effect of decreased bath $[Ca^{2+}]$ on insulin-mediated glucose uptake in whole EDL muscles. However, with a resting $[Ca^{2+}]_i$ of ~50 nM and a membrane potential of -80 mV in skeletal muscle cells, the extracellular $[Ca^{2+}]$ concentration has to be <1 nM before the inward driving force for Ca^{2+} is completely abolished (Bruton *et al.*, 1999). Thus, it is doubtful whether the extracellular $[Ca^{2+}]$ in isolated whole muscles can be decreased to levels low enough to affect insulin-induced Ca^{2+} uptake and hence glucose transport. We therefore used fluorescent 2-DG, 2-NBDG, to assess glucose uptake in single FDB fibers. Under control conditions insulin increased glucose uptake about two-fold (Figure 3C, *paper III*). The insulin-mediated increase in 2-NBDG fluorescence was reduced by ~40% when cells were exposed to low $[Ca^{2+}]$ (~1 nM) as compared to normal $[Ca^{2+}]$ (1.8 mM) (Figure 3A-C, *paper III*).

Application of 2-APB (100 µM) decreased the insulin-induced increase in 2-NBDG uptake by ~75% (Figure 3C, *paper III*). Moreover, the insulin-mediated increase in 2-NBDG fluorescence was reduced by ~80 % in the TRPC3 knock down fibers compared to control (Figure 4A, *paper IV*). Basal 2-NBDG uptake was not significantly different in TRPC3 knock down fibers compared to that measured in control fibers (data not shown).

OAG potentiates insulin-mediated glucose uptake both in WT and ob/ob fibers

Insulin plus OAG increased the Ca^{2+} influx above that observed by insulin alone (Figure 1E, *paper III*), and accordingly exposure to insulin and OAG gave an increase in 2-NBDG fluorescence that was about twice as large as that of insulin alone ($P < 0.01$, Figure 3C, *paper III*). Measurement of 2-DG uptake in whole EDL muscles exposed to insulin plus OAG also resulted in a significantly ($P < 0.01$) larger uptake (98.7 ± 5.7 µM min^{-1}) compared to insulin alone (79.0 ± 3.0 µM min^{-1}).

In single FDB fibers from WT mice, insulin resulted in an ~2-fold increase in 2-NBDG uptake compared to an ~1.2-fold in *ob/ob* fibers (Figure 4C-D, *paper III*), which shows that

the *ob/ob* fibers were insulin resistant. Nevertheless, as in WT fibers, exposure of *ob/ob* fibers to insulin plus OAG resulted in an increase in 2-NBDG uptake that was about twice as large as that of insulin alone (Figure 3C, *paper III*).

The effect of 2-APB on basal, insulin-, hypoxia-, and contraction-mediated glucose uptake in whole muscles

Basal glucose transport in skeletal muscle is mainly mediated by the GLUT1 transporter, which is constitutively present in the plasma membrane (Mueckler, 1994). Basal 2-DG uptake in EDL and soleus muscles was not significantly affected by 2-APB or OAG (Figure 5A, *paper III*). Thus, changes in Ca^{2+} influx appear to have no effect on basal glucose uptake. Application of 2-APB resulted in a dose-dependent decrease in insulin-mediated 2-DG uptake in mouse fast-twitch EDL and slow-twitch soleus muscles (Fig. 4A-B, *paper III*). In whole EDL muscles, application of 2-APB (100 μM) approximately halved the 2-DG uptake in the presence of insulin ($P < 0.01$). The inhibitory effect of 2-APB on insulin-mediated 2-DG uptake was not restricted to mouse muscle, since similar results were obtained in rat EDL and soleus muscles (Figure 4C, *paper III*). Thus with 100 μM 2-APB, insulin-mediated 2-DG uptake was decreased by about 50% in fast- and slow-twitch muscles of mouse and rat.

Hypoxia and repeated contractions are known to increase glucose uptake in skeletal muscle by pathways that differ from that of insulin (Holloszy, 2003;Holloszy & Narahara, 1967). Hypoxia (induced by exposing muscles to N_2) resulted in about a two-fold increase in 2-DG uptake that was not affected by 2-APB (Figure 5B, *paper III*). Repeated tetanic contractions for 10 min increased 2-DG uptake about three-fold in EDL muscles both in the presence and absence of 2-APB (Figure 5C, *paper III*). Thus, while the 2-APB-sensitive Ca^{2+} influx is important for insulin-mediated glucose uptake, other modes of activating glucose uptake are not affected by 2-APB.

The effects of 2-APB or TRPC3 knock down on tetanic force and $[Ca^{2+}]_i$

Stimulation of adult muscle fibers with brief electrical pulses results in action potential-mediated Ca^{2+} release from the SR, increased $[Ca^{2+}]_i$, activation of contractile filaments, and eventually force production (Allen *et al.*, 2008;Melzer *et al.*, 1995). In isolated FDB fibers (*paper III*) tetanic force and $[Ca^{2+}]_i$ were measured before and immediately after or during

exposure to 2-APB. Mean data showed a tetanic force of 100 ± 5% and $[Ca^{2+}]_i$ of 99 ± 10% of that before 2-APB exposure (n = 6). In whole muscles exposed to 2-APB (100 μM) for 30 min, tetanic force was marginally affected showing an increase to 114 ± 4% in soleus (n = 5; $P < 0.05$) and 107 ± 5% in EDL muscles (n = 4; $P < 0.05$), and the relaxation speed was slightly decreased. Thus, 2-APB has little effect on action potential-mediated SR Ca^{2+} release and force production.

During single 70 Hz tetanic stimulations $[Ca^{2+}]_i$ was similar in fibers cultured with (1.12 ± 0.06 μM, n = 9) or without (1.30 ± 0.08 μM, n = 9) TRPC3-siRNA for 48 hours (Figure 3A, *paper IV*). Tetanic force can not be measured in enzymatically dissociated TRPC3 knock down fibers. Thus, the CNT-induced transfection and knock down of TRPC3 had no effect on action potential-induced SR Ca^{2+} release.

The effects of APB or TRPC3 knock down on insulin signaling

To determine whether 2-APB or knock down of TRPC3 interfered with the insulin signaling pathway, the phosphorylation state of Akt was measured. Insulin increased Akt phosphorylation and this increase was not affected by 2-APB (Figure 6A, *paper III*) or by knock down of TRPC3 (Figure 3B, *paper IV*). These findings demonstrate that neither 2-APB nor the level of TRPC3 expression interferes with early insulin signaling steps in the pathway that terminates with GLUT4 translocation and glucose uptake (Bryant *et al.*, 2003;Huang & Czech, 2007). Furthermore, insulin is involved in cell proliferation via phosphorylation of the ERK1/2 (Saltiel & Kahn, 2001). Insulin increased ERK1/2 phosphorylation and this effect was not significantly altered by the presence of 2-APB (Figure 6B, *paper III*), indicating that 2-APB does not interfere with early signaling steps in the branch that results in cell proliferation.

Protein-protein interaction between TRPC3 and GLUT4

We first presented findings *(paper III)* that pharmacological inhibition of Ca^{2+} influx is associated with decreased insulin-mediated glucose uptake. These results were followed by results demonstrating that knock down of TRPC3 protein inhibited OAG-induced Ba^{2+} influx as well as insulin-mediated glucose uptake *(paper IV)*. Previous studies have shown that both TRPC3 and GLUT4 translocate to the surface membrane in response to agonist stimulation

(Dugani & Klip, 2005;Khan & Pessin, 2002;Singh *et al.*, 2004;Smyth *et al.*, 2006). In addition, in cardiomyocytes we showed an insulin-induced translocation of TRPC3 (Figure 6D, *paper II*). We therefore studied the possibility of a protein-protein interaction between TRPC3 and GLUT4 in adult muscle fibers. Figure 4B (*paper IV*) shows that TRPC3 co-immunoprecipitates with GLUT4 both in the presence and absence of insulin, which implies that they physically interact and move together upon insulin stimulation. To establish the specificity of the TRPC3-GLUT4 interaction, we performed control experiments with TRPC4. TRPC4 belongs to another TRPC subgroup, which is expressed and detected in skeletal muscle (data not shown, (Vandebrouck *et al.*, 2002)) but not activated by DAG (Venkatachalam *et al.*, 2003;Plant & Schaefer, 2005;Plant & Schaefer, 2003), and hence would not be involved in insulin signaling. Accordingly, TRPC4 was not detected in GLUT4 immunoprecipitates (Figure 4B, lower panels, *paper IV*).

TRPC3 and GLUT4 are co-localized in the t-tubular system of adult skeletal muscle

TRPC3 and GLUT4 immunofluorescence staining showed a distinct striated pattern perpendicular to the long axis of freshly isolated, relaxed muscle fibers (Figure 4C and D, *paper IV*). The staining of the two proteins overlaps (Mander's overlap coefficient = 0.80 (Li *et al.*, 2004)) indicating co-localization (Figure 4E, *paper IV*). The distance between the centers of two adjacent bands was ~2.05 μm, which is close to the sarcomere length in non-stretched, relaxed muscle fibers (Huxley & Niedergerke, 1954). In mammalian fibers, there are two t-tubules per sarcomere and these are located at the A-I band interface. The length of the A band is constant at 1.6 μm and hence the distance between two adjacent t-tubules is ~0.45 μm and this short distance makes it difficult to see the pairs of t-tubules in a sarcomere. Nevertheless, image analysis revealed double peaks at the expected distance (Figure 4F, *paper IV*). Thus, TRPC3 and GLUT4 co-localize in the proximity of the t-tubular system, which is considered to be the predominant site for glucose uptake in skeletal muscle (Khan *et al.*, 2001;Lauritzen *et al.*, 2006;Munoz *et al.*, 1995;Marette *et al.*, 1992;Wang *et al.*, 1996).

DISCUSSION

A general discussion regarding *papers I-IV* will follow; for more detailed discussions see the individual papers.

Paper I

Altered Ca^{2+} handling in *ob/ob* cardiomyocytes

Effects of insulin and IP_3 on cytoplasmic Ca^{2+} transients

In WT cardiomyocytes, application of insulin increased the Ca^{2+} transient amplitude and this inotropic effect may, to some extent, be explained by increased L-type Ca^{2+} currents (Aulbach *et al.*, 1999;Maier *et al.*, 1999). Conversely, in *ob/ob* cardiomyocytes insulin stimulation had no effect on the Ca^{2+} amplitude, but resulted in slowed Ca^{2+} transients and the appearance of frequent extra Ca^{2+} transients. Such events were never observed in WT cells under any conditions. This indicates an inability of *ob/ob* cells to increase the L-type Ca^{2+} current in response to insulin. Instead insulin appeared to prolong the SR Ca^{2+} release process in *ob/ob* cells, whereas it had little effect on Ca^{2+} removal, which is dominated by active SR Ca^{2+} reuptake (Bers, 2002). This argument is supported by the finding that application of 30 μM 2-APB, which under these conditions inhibits IP_3-mediated Ca^{2+} release (Maruyama *et al.*, 1997;Hamada *et al.*, 1999;Gysembergh *et al.*, 1999;Peppiatt *et al.*, 2003;Ascher-Landsberg *et al.*, 1999), inhibited the insulin effect in *ob/ob* whereas it had no effect on the insulin-mediated increase in Ca^{2+}amplitude in WT cells. Application of a membrane-permeant IP_3 analogue gave quantitatively the same results as those of insulin in *ob/ob* cardiomyocytes. This also suggested a role of IP_3 in insulin signaling in *ob/ob* cells. Thus, both insulin and IP_3 apparently increased the duration of action potential-mediated SR Ca^{2+} release in *ob*/*ob* cells and this was accompanied by the triggering of extra Ca^{2+} transients. The occurrence of extra Ca^{2+} transients may induce extra heart beats, indicating an increased risk for developing arrhythmias.

Possible role of IP_3 in insulin signaling and arrhythmia

IP_3 is shown to induce cardiac arrhythmias (Jacobsen *et al.*, 1996;Zima & Blatter, 2004;Mackenzie *et al.*, 2002) and insulin administration is shown to increase DAG levels (Romero *et al.*, 1988;Farese, 2001;Farese *et al.*, 1988;Eichhorn *et al.*, 2001). Since PLCγ mediated DAG production is associated with a stochiometric IP_3 production, the insulin dependent arrhythmias in *ob/ob* cardiomyocytes could derive from increased IP_3 production. Indeed, application of insulin caused an ~30% increase in the IP_3 concentration in *ob/ob* cardiomyocytes, albeit no detectable effect was found in WT cells. Generally IP_3-induced Ca^{2+}-release is considered to occur via a mechanism of action where generated IP_3 will bind to, and activate, IP3-receptors located on SR. This leads to opening of the receptors that are Ca^{2+} release channels, resulting in increases in intracellular Ca^{2+} (Berridge, 1993;Berridge *et al.*, 2003). The expression of IP_3 receptor isoform 1 and 2 was not different between *ob/ob* and WT ventricles and hence cannot explain the differences between the two groups regarding the response to insulin and IP_3. In addition, pre-incubation with 2-APB prevented the insulin and IP_3 induced effects on Ca^{2+} transients, further supporting a role of IP_3 in insulin signaling in *ob/ob* cardiomyocytes. We recognize that 2-APB can inhibit both Ca^{2+} influx and IP_3-induced Ca^{2+}-release (Bootman *et al.*, 2002;Iwasaki *et al.*, 2001;Prakriya & Lewis, 2001;Ma *et al.*, 2003;Ma *et al.*, 2002;Maruyama *et al.*, 1997;Ascher-Landsberg *et al.*, 1999;Peppiatt *et al.*, 2003). Therefore, it is possible that the removal of extra Ca^{2+} transients by 2-APB could be due to inhibition of Ca^{2+} influx instead of IP_3-induced Ca^{2+}-release, or inhibition of both processes. Regardless of the Ca^{2+} source, insulin and IP_3 induced extra Ca^{2+} transients in *ob/ob* cardiomyocytes. These may predispose for arrhythmia, which is a large problem in insulin resistant conditions including type 2 diabetes (Nesto, 2004;Fang *et al.*, 2004).

Reduced mitochondrial function may contribute to defective intracellular Ca^{2+} handling in ob/ob cardiomyocytes

Mitochondria are described as power plants since they generate a majority of the ATP in the cell, which is the form of energy that cells utilize. Mitochondria are also involved in intracellular Ca^{2+} handling and a site for ROS production (McCormack *et al.*, 1990;Duchen, 2000;Franzini-Armstrong, 2007;Robert *et al.*, 2001;Reid, 2001). In addition, mitochondrial dysfunction is often associated with type 2 diabetes (Levy, 1999;Lowell & Shulman, 2005;Duchen, 2004;Brownlee, 2005;Mazumder *et al.*, 2004). In *paper I*, we observed slowed mitochondrial Ca^{2+} uptake in *ob/ob* cells compared to WT cells. Moreover, in *ob/ob* cells Ca^{2+}

uptake was not increased by application of insulin or IP_3, indicating decreased mitochondrial Ca^{2+} buffering capacity. Thus, the impaired mitochondrial Ca^{2+} uptake in *ob/ob* cells may contribute to the larger slowing of insulin-and IP_3-induced Ca^{2+} transients, as well as the occurrence of extra Ca^{2+} transients. Furthermore, impaired mitochondrial Ca^{2+} accumulation is known to have deleterious effects by increasing $[Ca^{2+}]_i$ (Jaconi *et al.*, 2000), which is associated with cardiac dysfunction and arrhythmias (Wehrens *et al.*, 2005;Heineke & Molkentin, 2006). Consequently, it is possible that with age *ob/ob* mice will develop further cardiac complications related to alterations in intracellular Ca^{2+} handling.

Papers II-IV

Ca^{2+} influx in cardiac and skeletal muscle

OAG-induced NSCCs were smaller in ob/ob compared to WT cells

OAG induced an NSCC, I_{OAG}, in both cardiac (*paper II*) and skeletal muscle (*paper IV*). This current could be blocked by pharmacological inhibitors, including Gd^{3+} and 2-APB, all of which are known to inhibit TRPC3 channels (Putney, Jr. *et al.*, 2001;Lievremont *et al.*, 2005;Trebak *et al.*, 2002). In addition, the I_{OAG} was inhibited when anti-TRPC3 antibodies were present in the patch pipette, which agrees with our hypothesis that the OAG-induced NSCCs are conducted through TRPC3 channels. Furthermore, the I_{OAG} was smaller in *ob/ob* cells compared to WT cells. The mechanism underlying the decreased I_{OAG} in insulin resistant *ob*/*ob* muscle cells is unclear. It was not due to decreased TRPC3 expression in skeletal muscle, although there was a tendency of decreased TRPC3 expression in *ob/ob* cardiomyocytes. Post-translational modifications have been associated with impaired TRPC3 function and this could be a possible explanation behind the decrease in I_{OAG} in *ob/ob* cells (Trebak *et al.*, 2005;Kwan *et al.*, 2006;Kwan *et al.*, 2004).

Obesity is associated with cardiac lipid accumulation that leads to increased amounts of lipid metabolites such as DAG (Christoffersen *et al.*, 2003;Timmers *et al.*, 2008) that could lead to DAG-induced NSCCs and hence a subsequent increase in $[Ca^{2+}]_i$. In *paper I*, we hypothesized that an increased $[Ca^{2+}]_i$ in *ob/ob* cardiomyocytes may lead to hypertrophy and cardiac failure. Conversely, we obtained smaller I_{OAG} in *ob/ob* cardiomyocytes, which may indicate an adaptive process to limit Ca^{2+} influx and hence reduce the risk of disturbances in $[Ca^{2+}]_i$ handling and the development of cardiomyopathy.

Insulin activated NSCCs but with characteristics different from I_{OAG}

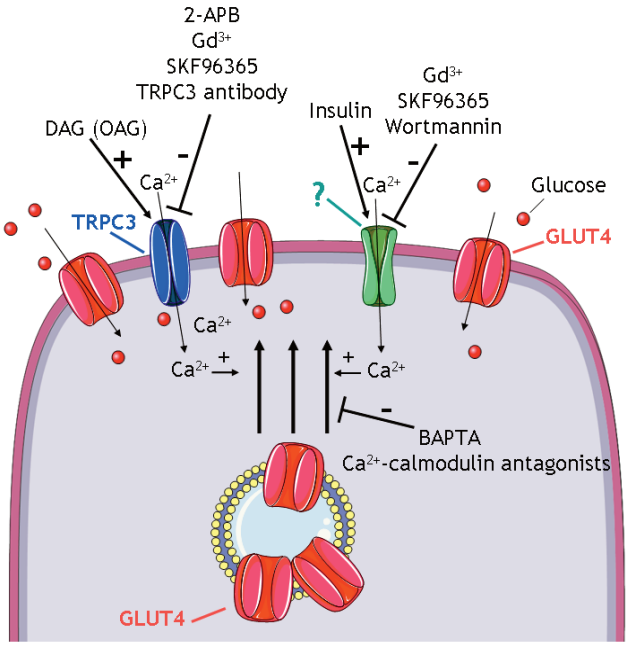
In cardiomyocytes, insulin activated an NSCC (*paper II*) with similar amplitude in WT and *ob/ob* cells and characteristics comparable to the insulin activated current reported by Zhang and Hancox (Zhang & Hancox, 2003). $I_{insulin}$ was also recorded in skeletal muscle fibers and there were no differences in current density between *ob/ob* and WT skeletal muscle fibers (data not shown). The $I_{insulin}$ exhibited characteristics that differed from those of I_{OAG}. For instance, neither 2-APB nor anti-TRPC3 antibodies inhibited $I_{insulin}$, indicating that this current is not mediated via TRPC3 channels. On the other hand, $I_{insulin}$ showed major similarities to the PI3K-dependent NSCC activated by insulin-like growth factor -1. Thus, $I_{insulin}$ may be mediated by the growth factor related channel, which is a murine homolog of the TRPV2 channel (Kanzaki *et al.*, 1999;Zhang & Hancox, 2003).

The extent of Ca^{2+} influx correlates with insulin-mediated glucose uptake

Skeletal muscle is quantitatively the major site for insulin-mediated glucose disposal in humans (DeFronzo *et al.*, 1985), and in *papers III and IV* we investigated the role of Ca^{2+} influx in insulin-mediated glucose uptake. We showed that manipulations that decreased Ca^{2+} influx also decreased insulin-mediated glucose uptake, whereas increased Ca^{2+} influx was associated with increased insulin-mediated glucose uptake. For instance, the presence of 2-APB, low extracellular [Ca^{2+}] or knock down of TRPC3 protein resulted in decreased Ca^{2+} influx and reduced insulin-mediated glucose uptake. On the other hand, application of OAG potentiated the Ca^{2+} influx and insulin-mediated increase in glucose uptake in isolated fibers and whole skeletal muscle from both WT and insulin resistant *ob/ob* mice.

Noteworthy is that solely changing Ca^{2+} influx is not sufficient to alter glucose transport because decreased (with 2-APB) or increased (with OAG) Ca^{2+} influx had no effect on glucose uptake in the absence of insulin, suggesting that insulin activates additional factors that are required for stimulation of glucose uptake. In addition, 2-APB only interfered with insulin-mediated glucose uptake; all other studied modes of glucose uptake (i.e. basal, hypoxia-, and contraction-mediated glucose uptake) were unaffected. Accordingly, basal glucose uptake was not different between TRPC3-siRNA treated fibers and the control conditions, which is consistent with the fact that basal glucose uptake does not involve GLUT4 translocation (Mueckler, 1994).

Taken together, these results demonstrate a complex interaction between basal, insulin- and OAG-induced Ca^{2+} influx and insulin-mediated glucose uptake, which indicates the involvement of several different ion channels (e.g. TRPC, TRPV2) or a complex regulation of one set of channels. Figure 3 shows a tentative model for the interaction between OAG (DAG)-mediated and insulin-mediated Ca^{2+} influx and GLUT4 translocation.



Adapted from Lanner *et al.* Curr Opin Pharmacol, 2008

Figure 3. ***Tentative model for interaction between DAG-mediated and insulin-mediated Ca^{2+} influx and GLUT4 translocation.*** The DAG effect appears to be mediated by TRPC3, whereas insulin works on a yet unidentified channel, although GRC/TRPV2 is a candidate. Antagonists of the respective channels are shown. Activation of the channels results in a localized increase in Ca^{2+} which probably facilitates docking/fusion/insertion of GLUT4 with the surface membrane. This process is blocked by the Ca^{2+} chelator BAPTA and by Ca^{2+}-calmodulin antagonists.

DAG appears to affect several intracellular targets

Elevated intracellular DAG levels have been observed in skeletal muscle of insulin-resistant rodents and humans (Itani *et al.*, 2002;Griffin *et al.*, 1999;Kraegen *et al.*, 2006;Yu *et al.*, 2002;Timmers *et al.*, 2008). Increased DAG levels have been suggested to induce insulin resistance and a tentative mechanism is increased conventional (α, β, γ) - and novel (δ, ε, η, θ)

PKC (c/nPKC)-mediated serine phosphorylation of the insulin receptor and the insulin receptor substrate-1 (Itani *et al.*, 2002;Griffin *et al.*, 1999;Kraegen *et al.*, 2006;Yu *et al.*, 2002;Shulman, 2000;Timmers *et al.*, 2008). Thus, DAG (OAG) may exert dual effects: acutely potentiate insulin-stimulated glucose uptake via increased TRPC3-mediated Ca^{2+} influx in a PKC-independent manner and in addition induce insulin resistance via a c/nPKC-mediated inhibition of early events in the insulin signaling cascade. How DAG can exert these opposing effects is unclear but could be related to DAG of different origins (e.g. PIP_2 hydrolysis, *de novo* synthesis), time course and cellular distribution of DAG. Thus, "different" DAGs could affect separate processes simultaneously. This could be a process partly similar to the toolkit Ca^{2+} uses, e.g. amplitude, duration and cellular localization to exert different effects on various cellular processes (Berridge *et al.*, 2003;Bootman *et al.*, 2001;Lewis, 2001).

Ca^{2+} influx involves TRPC3 in a late step of the insulin signaling cascade

Phosphorylation of Akt is considered to be an important step in the insulin signaling cascade leading to glucose uptake. In contrast to OAG-induced Ca^{2+} influx and insulin-stimulated glucose uptake, Akt phosphorylation was not affected by 2-APB or TRPC3 knock down, which indicates that the role of Ca^{2+} is downstream of Akt.

Decreased TRPC3 protein expression by ~40% resulted in decreased insulin-mediated glucose uptake by ~80%. Biological processes that involve hydrophilic hormones (e.g. insulin, catecholamines) and intracellular signaling often are cascade reactions, i.e. the signals diverge several times. Thus the changes in TRPC3 expression, Ca^{2+} influx and glucose uptake do not need to be stoichiometric. In addition, TRPC3 channels appear to be present at the plasma membrane and in intracellular vesicles and the channels involved in Ca^{2+} influx, would be the fraction inserted in the plasma membrane. It should be noted that we measured the total decrease in TRPC3 expression and not the membrane fraction. Taken together, the data suggest that Ca^{2+} influx through TRPC3 plays an important role in a late step in the insulin signaling cascade leading to glucose uptake.

TRPC3 and GLUT4 co-localize in striated muscle

The t-tubular system is considered to be the predominant site for insulin-mediated glucose transport in skeletal muscle (Khan *et al.*, 2001;Lauritzen *et al.*, 2006;Munoz *et al.*, 1995;Marette *et al.*, 1992;Wang *et al.*, 1996). In cardiomyocytes, the GLUT4 also translocates to the t-tubular system in response to insulin (Slot *et al.*, 1991). Immunofluorescence staining (*paper IV*) showed that TRPC3 and GLUT4 co-localize in the proximity of the t-tubules. Conversely, TRPC3 staining in cardiomyocytes (*paper II*) showed a different pattern, with localization directly on as well as immediately beneath the sarcolemma. This may indicate that the channels have different functions in the separate tissues. Nevertheless, an association between TPRC3 and GLUT4 was detected with immunoprecipitation both in skeletal and cardiac muscle, both in the absence and presence of insulin.

GLUT4 is found to assemble in vesicles as part of a larger protein complex. In response to insulin, these vesicles translocate to the plasma membrane and undergo docking and fusion (Khan *et al.*, 2001;Martin *et al.*, 1996;Cheatham, 2000;Foster & Klip, 2000), i.e. a process similar to the Ca^{2+}-dependent vesicle fusion in synaptic terminals (Burgoyne & Morgan, 1993;Schneggenburger & Neher, 2005). The GLUT4-containing vesicles contain VAMP2 and synaptophysin, which are v-SNARE proteins that interact with t-SNARE proteins at the surface membrane (Khan *et al.*, 2001;Martin *et al.*, 1996;Min *et al.*, 1999;Okada *et al.*, 2007). TRPC3 is also found in larger protein complexes, which contain proteins that are also found in the GLUT4-containing vesicles (e.g. VAMP2 and synaptophysin) (Singh *et al.*, 2004) and both TRPC3 and GLUT4 translocate to the plasma membrane in an agonist-induced manner (Singh *et al.*, 2004;Smyth *et al.*, 2006) . This suggests that TRPC3 and GLUT4 are members of a larger protein complex that translocate together.

Taken together, we propose the following model (*papers II-IV*): Ca^{2+} influx modulates a late step of the insulin-dependent recruitment of GLUT4 to the plasma membrane (Inoue *et al.*, 1999;Whitehead *et al.*, 2004). GLUT4 and TRPC3 are components of the same protein complex and hence move together in response to insulin. Furthermore, reduced Ca^{2+} influx via TRPC3, in the presence of insulin, causes a decrease in glucose transport, whereas an OAG treatment in the presence of insulin increases Ca^{2+} influx and potentiates insulin-mediated glucose uptake. Thus, TRPC3 is a potential target for treatment of conditions associated with insulin resistance, including type 2 diabetes.

GENERAL CONCLUSIONS

- In *ob/ob* cardiomyocytes, insulin and IP_3 revealed defects in intracellular Ca^{2+} handling that can increase the probability of extra Ca^{2+} transients and may predispose for arrhythmias. On the other hand, decreased current density in TRPC3 mediated DAG-induced NSCCs was observed , which could be a compensatory mechanism to reduce the possible harmful effects of prolonged increases in $[Ca^{2+}]_i$ in cardiomyocytes.

- Ca^{2+} influx plays an important role in insulin-mediated glucose uptake in skeletal muscle. GLUT4 and TRPC3 co-localize in the t-tubular system and are components of the same protein complex and hence move together. TRPC3 is a potential target for treatment of conditions associated with insulin resistance.

PROSPECTIVES

Cardiomyocyte function:

A major goal is to prevent cardiomyopathy associated with obesity and insulin resistant conditions. To achieve this goal increased understanding of cardiac function and dysfunction is needed. For instance, insulin resistance is associated with hyperinsulinemia and we therefore need to elucidate what channel(s) insulin activates, how they interact with TRPC3, and what consequences this has for cardiac function. Moreover, DAG appears to have several intracellular targets. We measured the short-term effects of DAG and in the future we need study how these relate to the long-term effects of DAG on cardiomyocyte function.

Ca^{2+} and glucose uptake:

To obtain further understanding of the molecular interaction of TRPC3 and GLUT4, fluorescence resonance energy transfer (FRET) and total internal reflection fluorescence (TIRF) would be useful. With these methods it is possible to detect protein-protein interaction in real time as well as movement of proteins over short distances. It appears that GLUT4 vesicles slow its movement in the presence of insulin to facilitate fusion with the surface membrane, whereas in the absence of insulin GLUT4 vesicles move too rapidly and this fusion rarely occurs (Lizunov *et al.*, 2005). There are features in this process that are conducive to the involvement of Ca^{2+} and with TIRF it would be interesting to examine what happens with GLUT4 trafficking if one manipulates with Ca^{2+} influx in the presence of insulin and/or DAG. These studies are likely to additional factors involved in these processes and needs to be identified to further elucidate the insulin-mediated GLUT4 trafficking leading to glucose uptake and the defects observed in insulin resistance conditions.

SVENSK SAMMANFATTNING

I dagens samhälle är övervikt och typ 2-diabetes (kallades tidigare åldersdiabetes eller icke-insulinberoende diabetes) växande hälsoproblem. Förutom arvsanlag är främsta orsakerna till dessa problem olämplig kost och brist på motion. Typ 2-diabetes är den vanligaste formen av diabetes och omfattar ~90% av alla diabetiker. I dagsläget beräknas att ~171 miljoner människor världen över har typ 2-diabetes; som jämförelse motsvarar detta ~35% av EU: s befolkning. En karakteristisk defekt vid övervikt och typ 2-diabetes är insulinresistens, vilket innebär att cellerna förmåga att reagera på insulin är nedsatt och därmed påverkas deras glukosupptag negativt. Detta kan leda till allvarliga komplikationer i flera organ, som njurar, nerver och hjärt-kärlsystemet. Enkel och effektiv behandling saknas för övervikt och typ 2-diabetes, detta tillsammans med det växande antalet personer med dessa problem medför kraftiga ekonomiska påfrestningar för hälso- och sjukvården. Insulinresistens samt associerade komplikationer kan vara relaterade till försämrad Ca^{2+} signalering i cellen. Ca^{2+} är involverat i många cellulära processer i vår kropp, som befruktning, kontraktion, gentranskription, hormon signalering, synapstransmission och cell död. För att kunna utföra dessa varierande processer är det viktigt att Ca^{2+} är strikt kontrollerat med avsikt på varaktighet, omfattning och lokalisation.

Denna avhandling fokuserar på interaktionen mellan Ca^{2+} och insulin i hjärt- och skelettmuskulatur. De första två artiklarna undersöker hur insulin påverkar Ca^{2+} homeostasen i hjärtceller från normala vildtypsmöss, samt hur detta skiljer sig i hjärtceller från en djurmodell som är överviktig samt insulinresistent, *ob/ob* möss. *Ob/ob* hjärtceller uppvisade förlängda Ca^{2+} transienter och försämrad mitokondriell Ca^{2+} hantering, vilket kan resultera i ökad risk för arytmier *in vivo*. *Ob/ob* cellerna visade även minskade jonströmmar via en ospecifik jonkanal, TRPC3, vilket kan leda till ytterligare förändrad cellulär Ca^{2+} balans. Skelettmuskulaturen är ett av de viktigaste målorganen för insulinstimulerat glukosupptag. De påföljande två artiklarna undersökte vilken uppgift Ca^{2+} har i insulinstimulerat glukosupptag i skelettmuskler. Vi fann en korrelation mellan mängden Ca^{2+} inflöde till skelettmuskeln och det insulinstimulerade glukosupptaget; ökat inflöde av Ca^{2+} resulterade i potentierat insulinstimulerat glukosupptag både i muskler från vildtypsmöss och *ob/ob* möss, medan minskat inflöde av Ca^{2+} ledde till minskat insulinstimulerat glukosupptag. Med hjälp av genteknik, så kallad siRNA, kopplad till nanotuber av kol kunde vi minska proteinuttrycket av

TRPC3. Detta resulterade i minskat agonistinducerat Ca^{2+} flöde och minskat insulinstimulerat glukosupptag. Insulinstimulerat glukosupptag sker via ett transportprotein, GLUT4 och vi kunde visa att GLUT4 och TRPC3 finns lokaliserade intill varandra i det område som anses vara den främsta platsen för glukosupptag i skelettmuskler. Detta tyder på att TRPC3 är en komponent i insulinsstimulerat glukosupptag.

Sammantaget har dessa studier gett en ökat kunskap om interaktionen mellan insulin och Ca^{2+} i hjärta och skelettuskulatur. I hjärtat identifierades jonkanaler och cellulära komponenter som medverkar till förändrad Ca^{2+} hantering och dessa kan därmed vara involverade i processer som orsakar hjärtkomplikationer kopplade till insulinresistens. Därtill visades att Ca^{2+} är viktigt för insulinstimulerat glukosupptag i skelettmuskel. I denna avhandling presenteras resultat som identifierar olika processer som kan fungera som mål för nya behandlingsmodeller vid insulinresistens och typ 2-diabetes.

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