University of Alberta

Metabolic Regulation of ATP-Sensitive Potassium Channels and Sodium/Calcium Exchange by Long Chain Acyl CoA Esters

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Edmonton, Alberta
Spring 2006

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ISBN: 0-494-14030-5
Our file Notre référence
ISBN: 0-494-14030-5

For my Grandfather and my Father, my inspiration and my motivation

ABSTRACT

The prevalence of type-2 diabetes is reaching pandemic proportions. Recent surveys estimate the current global incidence of type-2 diabetics at greater than 170 million with this number expected to double in the next twenty-five years. The etiology of type-2 diabetes involves environmental, lifestyle, and genetic components, which combine to result in insulin resistance and an ultimate decrease in glucose-stimulated insulin secretion. Among the leading risk factors for the development of this debilitating condition is obesity. In fact, the incidence of these two conditions has paralleled one another throughout recent history and approximately 80% of type-2 diabetics are considered over-weight or obese.

As a consequence of obesity, plasma free fatty acid levels increase leading to cellular accumulations of lipids and metabolic intermediates including long chain acyl CoA esters. These fatty acid metabolites are known regulators of several proteins including ion channels. As our understanding of the inner workings of the pancreatic insulin-secreting β-cell has developed, so too has our ability to identify proteins that might be dysfunctional in the diabetic individual. Among these are ATP-sensitive potassium (K_{ATP}) channels, involved in coupling metabolism to electrical excitability in such a manner as to control the timely release of insulin, and the sodium/calcium exchanger, involved in regulating intracellular calcium homeostasis.

Although the genetic component of common type-2 diabetes is not well understood, increasing numbers of K_{ATP} channel mutations and polymorphisms are being described that result in β-cell electrical dysfunction or increased susceptibility to type-2

diabetes. Of particular interest is the E23K polymorphism, which increases susceptibility to type-2 diabetes in Caucasian populations and may also be associated with weight gain and obesity.

It is the aim of this thesis to report my findings that examined a model of elevated circulating fatty acid levels on electrical excitability and ultimately function of the insulin-secreting pancreatic β-cell. Focus is placed on the possible molecular mechanisms by which polymorphic K_{ATP} channels and the sodium/calcium exchanger modulate β-cell electrical excitability in the presence of elevated intracellular acyl CoAs. The results presented in this thesis have implications for both type-2 diabetes as well as cardiac pathologies such as ischemia/reperfusion injury.

ACKNOWLEDMENTS

The work presented in this thesis could not have been completed without the assistance of a number of very important individuals. I would like to first thank my supervisor and mentor, Dr. Peter Light for allowing me the opportunity to study in his laboratory. The experience I have gained while working with him has provided me a solid foundation on which to start a meaningful career. I am also indebted to several current and past members of the lab who have contributed to my growth as a scientist. Dr. István Baczkó and Dr. Jocelyn Manning Fox have been particularly instrumental in my scientific development and I look forward to continued collaborations and long-lasting friendship. Diana Steckley, Matthew Fercho, and especially Lynn Jones have provided expert technical assistance throughout my studies and I thank them for their help.

To the members of my committee, Dr. Martin Davies and Dr. Rhonda Bell, I thank you for you intellectual contributions, encouragement, and guidance during my studies. I would also like to thank Dr. Joseph Bryan and Dr. Peter Smith for participating in my thesis defense.

Many people have contributed directly to my work. I thank Dr. Gerda de Vries for providing us with the β-cell mathematical modeling presented in this work. I would also like to thank Suzanne Kovacic and Dr. Jason Dyck for supplying viruses and neonatal cardiomyocyte preparations for our cardiac sodium/calcium exchange project. Finally, I thank Ken Strynadka for his assistance with our HPLC experiments.

My thesis work has been supported by a number of funding agencies to whom I am very grateful. I would like to thank the Alberta Heritage Foundation for Medical Research and the Canadian Diabetes Foundation for providing my stipend throughout the course of my doctorate. I would also like to thank the Canadian Institutes of Health Research for funding many of the projects undertaken during my studies.

Finally, I would like to thank my wife, Jane and my mother, Sylvia for enduring the ups and downs of my degree with me and for providing me with the motivation and support I have needed to complete this work.

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LIST OF ABBREVIATIONS AND SYMBOLS

$[ATP]_i$	Intracellular ATP concentration
$[Ca^{2+}]_{i/o}$	Intracellular (i) / Extracellular (o) calcium concentration
$[Na^{+}]_{i/o}$	Intracellular (i) / Extracellular (o) sodium concentration
AbXIP	Anti-XIP antibody
Acyl CoA	Acyl Co-enzyme A ester
ADP	Adenosine diphosphate
AMPK	Adenosine monophosphate-regulated protein kinase
ATP	Adenosine triphosphate
BMI	Body Mass Index
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
cDNA	Complimentary deoxyribonucleic acid
CO_2	Carbon dioxide
CoA	Co-enzyme A
CPT-1	Carnitine-Palmitoyl Transferase-1
DHA	Docosahexaenoic acid
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethylsulfoxide
E/E	Homozygous wild-type genotype of E23K
E/K	Heterozygous polymorphic genotype of E23K
E23K	Glutamic acid to lysine mutation at residue 23 in Kir6.2

E_{Ca}	Equilibrium potential for calcium
EGTA	Ethylene glycol bis(2-aminoethyl ether)-n,n,n,n-tetraacetic acid
E_{Na}	Equilibrium potential for sodium
$E_{Na/Ca}$	Reversal potential for sodium/calcium exchange
EPA	Eicosapentaenioc acid
F	Faraday's Constant = 9.6485 X 10^4 C mol^{-1}
F255E	Phenylalanine to glutamic acid mutation at residue 255 in NCX
FACS	Fatty acyl Co-enzyme A synthetase
FFA	Free fatty acid
GDP	guanosine 5'-diphosphate
GLP-1	Glucagon-like peptide-1
GPR40	40 KDa G-protein-coupled receptor
GSIS	Glucose stimulated insulin secretion
HEPES	4-(hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
I_1 inactivation	Sodium-dependent inactivation
I337V	Isoleucine to valine mutation at residue 337 in Kir6.2
IC_{50}	Half-maximal concentration required for channel inhibition
IRP	Immediately releasable pool
K/K	Homozygous polymorphic genotype of E23K
K^+	Potassium
K_{ATP} channel	ATP-sensitive potassium channel
KcsA	Bacterial potassium channel from Streptomyces coelicolor A3(2)

Kir6.2	Inward rectifier potassium channel subunit, isoform 6.2
Kir6.2ΔC26	Truncation mutation in Kir6.2 that removes the endoplasmic reticulum retention signal found within the first 26 C-terminal residues
Kirbac1.1	Bacterial inward rectifier potassium channel, isoform 1.1
L270V	Lysine to valine mutation at residue 270 in Kir6.2
Li^+	Lithium
Log P	Partition coefficient between octanol and water
MODY	Maturity Onset Diabetes of the Young
MTS	methanethiosulfonate
Na^+	Sodium
NBF	Nucleotide binding fold
NCX	Sodium/calcium exchanger
pA	picoAmps
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Pf	Plateau fraction
pfu	Plaque-forming units
PHHI	Persistent Hyperinsulinemic Hypoglycemia of Infancy
PIP	Phosphatidylinositol 4-phosphate
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
P_o	Open probability

$P_{o,zero}$	Spontaneous open probability in the absence of ATP
PPARγ	Peroxisome proliferator-activated receptor, isoform γ
Pro12Ala	Proline to alanine mutation at residue 12 in PPARγ
pS	picoSiemens – measure of conductance
R	Gas constant = 8.3145 J mol^{-1} K^{-1}
R201H	Arginine to histidine mutation at residue 201 in Kir6.2
SE	Standard error
SUR	Sulphonylurea receptor
T	Temperature in ° Kelvin
TCF7L2	Transcription factor 7-like 2
TEA-Cl	Tetraethylammonium chloride
V_{max}	Maximum slope of activation
VNTR	Variable number of tandem repeats
WT	Wild-type
XIP	Exchanger inhibitory peptide
τ	Tau

Chapter 1

General Introduction

Portions of this chapter have been previously published in *Human Genetics* under the following citation:

Riedel,M.J., Steckley,D.C., and Light,P.E. (2005). Current status of the E23K Kir6.2 polymorphism: Implications for type-2 diabetes. Human Genetics *116*(3):133-145.

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The prevalence of type-2 diabetes is reaching pandemic proportions throughout the developed world. There are currently more than 170 million affected individuals world-wide and that number is expected to double by the year 2030 (Wild et al., 2004). The rapid rise in type-2 diabetes has been mirrored by a rise in the incidence of obesity, highlighting increased weight gain as a major risk factor for the development of this disease. Both type-2 diabetes and obesity are important risk factors for the development of a number of diseases including cardiovascular disease, cancer, and stroke.

Obesity results from an imbalance between caloric intake and energy expenditure. Excess calories from both carbohydrates and fat can contribute to increased adiposity if energy expenditure is not sufficient. With the prevalence of obesity continuing to rise (Katzmarzyk, 2002), both in adult and child populations (Tremblay et al., 2002), it is important that we understand the mechanisms by which increased caloric intake, energy storage, and nutrient metabolism can affect important physiological processes and the development of disease.

Fatty acids and their intracellular metabolites can act as signaling molecules, modulating gene expression as well as the function of enzymes, ion channels and other membrane-bound and soluble proteins (Nakamura et al., 2004; Hulbert et al., 2005). However, the cellular and molecular mechanisms that underlie these signaling mechanisms are not fully characterized. To elucidate these mechanisms, it is first required that we understand the cellular physiology of affected tissues and the way in which that physiology may be altered in these disease states.

Much work has been done in the last 50 years to piece together the physiological puzzle that underlies such processes as insulin secretion from pancreatic β-cells and

excitation-contraction coupling in cardiomyocytes. With a fundamental understanding of these processes in place, the focus of more recent research has been on potential modulators of these physiological processes that may contribute to disease onset and progression. In this respect, there has been significant interest in the role of lipids and lipid metabolism in regulating physiological and pathophysiological processes. In particular, the work presented in this thesis will examine the effects of fatty acid intermediates known as acyl CoA esters (acyl CoAs), which are known regulators of various proteins, on ATP-sensitive potassium (K_{ATP}) channels and the sodium/calcium exchanger. This work has direct implications for understanding increased caloric intake, dyslipidemia, and obesity as risk factors for type-2 diabetes and as potential contributors to cardiac dysfunction sustained during ischemia/reperfusion injury.

Section 1: K_{ATP} Channel Studies

β-Cell Electrical Excitability and Nutrient-Stimulated Insulin Secretion

K_{ATP} Channel-Dependent Glucose Stimulated Insulin Secretion

The strict regulation of plasma glucose levels is a critical physiological process. Impaired glucose homeostasis can lead to severe physical and mental damage and premature death as seen in the pathogenesis of diseases such as persistent hyperinsulinemic hypoglycemia of infancy (PHHI) and diabetes mellitus. To maintain plasma glucose levels within the narrow physiological range (3.8 – 11.1 mM), the body has developed a system whereby the secretion of hormones that control either the uptake of glucose from the plasma or release of glucose into the plasma can be fine-tuned as per physiological need. Insulin is the primary hormone responsible for catalyzing the uptake and storage of glucose into various tissues and is secreted from pancreatic β-cells

primarily in response to glucose, but also in response to other nutrients such as fatty acids and certain amino acids. As post-prandial plasma glucose levels rise, increased glycolysis and glucose oxidation in the β-cell leads to ATP formation at the expense of ADP. The increase in cytosolic ATP-to-ADP ratio results in the closure of K_{ATP} channels and membrane depolarization via reduced potassium efflux (Ashcroft et al., 1984; Ashcroft and Gribble, 1999). Subsequent activation of voltage-gated calcium channels is followed by transient increases in intracellular calcium, which then trigger the exocytosis of insulin-containing granules (Ashcroft and Rorsman, 1989; Figure 1-1). While other ion channels such as voltage-gated (MacDonald and Wheeler, 2003) and calcium-activated potassium channels (Ashcroft and Rorsman, 1989) are involved in repolarizing the membrane potential, K_{ATP} channel activity serves to transduce the glucose-mediated metabolic signal into alterations in electrical activity that initiate insulin secretion. This mechanism of K_{ATP} channel closure-dependent initiation of secretion is shared by the glucagon-like peptide-1 (GLP-1)-secreting intestinal L-cell (Reimann and Gribble, 2002), but not by the glucagon-secreting pancreatic α-cell (Ashcroft, 2000). Indeed, in the α-cell, K_{ATP} channel inhibition is associated with reduced glucagon secretion. In these cells, a larger sodium channel conductance coupled with a smaller K_{ATP} channel conductance sets up a mechanism by which elevated glucose suppresses glucagon secretion (Gopel et al., 2000). As in the β-cell, glucose metabolism in the α-cell increases intracellular ATP concentrations. However, the subsequent K_{ATP} channel closure and membrane depolarization leads to inactivation of sodium channels and a reduction in electrical excitability and glucagon secretion (Gopel et al., 2000). Therefore, the same stimulus that increases insulin secretion from β-cells causes a reduction in

glucagon secretion from α-cells. Likewise, mechanisms that involve activation of the K_{ATP} channel can lead to impaired insulin secretion and enhanced glucagon secretion, potentially exacerbating hyperglycemia.

Several other stimuli can elicit insulin secretion either in the absence of stimulatory glucose concentrations or as an amplifying signal to boost insulin secretion only when sufficient glucose levels are first present. Among these stimuli are cholinergic innervation, amino acids, and hormones including GLP-1. GLP-1 is a hormone released from intestinal L-cells that acts at many targets within the β-cell to increase glucose-stimulated insulin secretion (for review, see MacDonald et al., 2002). Interestingly, one target of action for GLP-1 in the β-cell is the cyclic adenosine monophosphate (cAMP)/PKA pathway. Through binding to its G-protein-coupled receptor on the plasma membrane, GLP-1 elicits an increase in cAMP formation, which in turn activates PKA. Subsequent phosphorylation of the K_{ATP} channel by PKA leads to modulation of channel activity. The way in which the K_{ATP} channel is modulated by PKA-dependent phosphorylation however, is dependent on the energy status of the cell. As such, under simulated low glucose conditions where the intracellular ATP-to-ADP ratio is low, PKA-mediated phosphorylation of the K_{ATP} channel results in a significant increase in channel activity. Conversely, under simulated high glucose conditions, the same phosphorylation event results in a significant decrease in channel activity (Light et al., 2002). Therefore, only in the presence of glucose is GLP-1 capable of significantly increasing β-cell electrical excitability (Figure 1-2) and subsequent insulin secretion.

Fat Metabolism and K_{ATP} Channels

In addition to the above mentioned stimuli, acute exposure of β-cells to free fatty acids results in increased insulin secretion via several proposed pathways. New evidence suggests the existence of a cell surface G-protein coupled receptor, GPR40, which may amplify glucose stimulated insulin secretion when bound by fatty acids through direct modulation of intracellular Ca^{2+} levels (Briscoe et al., 2003; Itoh et al., 2003). In addition, free fatty acids can diffuse or be actively transported across the plasma membrane. During periods of elevated plasma fatty acids, a concomitant increase in cytosolic fatty acids can occur. One potential mechanism for the stimulation of insulin secretion under these conditions is a direct inhibition of K_{ATP} channel activity by these non-esterified fatty acids (Figure 1-3). The importance of this mechanism has not been fully characterized as most free fat that enters the β-cell is esterified to coenzyme A by fatty acyl CoA synthetase (FACS), forming acyl CoAs (Corkey et al., 2000; Suzuki et al., 1990; Coleman et al., 2002). These lipid metabolites consist of a hydrocarbon tail corresponding to the respective free fatty acid, esterified via a thiol-ester linkage to co-enzyme A. This final product is therefore an amphiphilic molecule containing a negatively charged, polar head group (CoA) and a hydrophobic tail. Interestingly, the structure of these molecules closely resembles that of ADP (Figure 1-4) and as such, CoA alone may act as a weak ADP analogue, relieving a portion of ATP inhibition and thus promoting a small increase in K_{ATP} channel activity (Fox et al., 2003). Under normal circumstances, these molecules are transported into the mitochondria and subsequently oxidized, forming ATP and thus promoting closure of K_{ATP} channels (Figure 1-1).

In direct contrast to non-esterified free fatty acids, acyl CoAs of carbon chain length greater than 12 can directly activate K_{ATP} channels (Branstrom et al., 1997; Branstrom et al., 1998; Gribble et al., 1998; Larsson et al., 1996). In individuals with elevated circulating levels of free fatty acids, including both obese individuals and those with type-2 diabetes, cytosolic acyl CoA levels can be increased (Golay et al., 1986; Reaven et al., 1988). This accumulation of acyl CoAs can occur as a result of directly increasing intracellular free fatty acid levels, but can also result from increased glucose metabolism. Excessive citrate production from the oxidation of glucose can lead to intracellular accumulation of acetyl CoA (Corkey et al., 2000). Conversion of the acetyl CoA to malonyl CoA by acetyl CoA carboxylase results in allosteric inhibition of carnitine-palmitoyl transferase-1 (CPT-1), the enzyme required to transport acyl CoAs in the form of acyl carnitines across the outer mitochondrial membrane. Therefore, in the presence of elevated glucose, reduced mitochondrial metabolism of fatty acids can lead to cytosolic accumulation of acyl CoAs. This suggests that chronic accumulation of acyl CoAs in β-cells, and their direct effects on K_{ATP} channel activity, may contribute to decreased glucose-stimulated insulin secretion (GSIS) and the development of type-2 diabetes.

Molecular Aspects of the K_{ATP} Channel

K_{ATP} channels of differing isoforms are expressed in a wide variety of tissues including the endocrine pancreas (Ashcroft, 2000; Bokvist et al., 1999), heart (Noma, 1983), intestine (Reimann and Gribble, 2002), and central nervous system (Karschin et al., 1998; Zawar et al., 1999). The distinctive properties of the K_{ATP} channel, as defined by its molecular composition, allow for the regulation of potassium (K^+) efflux by

intracellular metabolites such as nucleotides and acyl CoAs. This creates a unique mechanism by which cellular metabolism can be coupled to alterations in electrical activity. The pancreatic K_{ATP} channel is a hetero-octomeric complex (Aguilar-Bryan et al., 1998; Clement et al., 1997; Shyng and Nichols, 1997) comprised of four inward-rectifier K^+ channel subunits (Kir6.2; Inagaki et al., 1995; Sakura et al., 1995) coupled to four high affinity sulphonylurea receptor subunits (Aguilar-Bryan et al., 1995; Figure 1-5). Kir6.2 is comprised of two transmembrane domains and a pore loop which forms the central K^+-conducting pore region of the channel. Both the short N-terminus and longer C-terminus are located on the cytosolic side of the plasma membrane (Figure 1-5).

The regulatory SUR subunit bestows the rich pharmacology upon the K_{ATP} channel complex and contains the high affinity binding domains for antidiabetic sulphonylurea drugs such as tolbutamide and glibenclamide as well as the K_{ATP} channel opener diazoxide used in the treatment of some forms of PHHI (Babenko et al., 2000; Huopio et al., 2002; Matsuoka et al., 2000; Uhde et al., 1999). The three SUR subunits so far identified are SUR1, SUR2A, and SUR2B. In recombinant systems, co-expression of SUR1 with Kir6.2 forms channels with properties identical to those channels found in pancreatic β-cells (Inagaki et al., 1995), α-cells (Bokvist et al., 1999), GLP-1-secreting L-cells (Reimann and Gribble, 2002), and certain neurons in the brain (Karschin et al., 1998; Zawar et al., 1999). K_{ATP} channels incorporating the SUR2A subunit are found in the sarcolemmal membrane of the heart as well as in skeletal muscle, while those incorporating SUR2B are found in vascular smooth muscle (For review, see Seino, 2003).

Both Kir6.2 and SUR1 contribute to the nucleotide sensitivity of the β-cell K_{ATP} channel. The Kir6.2 subunit confers ATP sensitivity to the channel complex (Aguilar-Bryan and Bryan, 1999; Tucker et al., 1997) while Mg-ADP interacts with the second nucleotide-binding fold (NBF-2) on SUR1, antagonizing ATP-mediated channel closure (Gribble et al., 1997; Nichols et al., 1996; Shyng and Nichols, 1997). Anionic lipid mediators such as acyl CoAs and phosphatidylinositol 4,5-bisphosphate (PIP_2) can further increase K_{ATP} channel activity through interaction with the Kir6.2 subunit (Branstrom et al., 1997; Larsson et al., 1996; Manning Fox et al., 2004; Schulze et al., 2003; Shyng and Nichols, 1998).

Genetic Variations in the K_{ATP} Channel and Diabetes Risk

Understanding the regulation of insulin secretion from the pancreatic β-cell has elucidated candidate genes such as glucokinase, hepatic nuclear factors-1α and 4α, and insulin promoting factor-1. Mutations in these genes produce dysfunctional proteins that contribute to the rarer monogenic maturity-onset diabetes of the young (MODY; Gloyn, 2003). However, identification of genetic mutations and/or polymorphisms contributing to the development of common type-2 diabetes has proved elusive and those discovered thus far have not been fully characterized. Until recently, the Pro12Ala polymorphism in the peroxisome proliferator-activated receptor (PPARγ; Rangwala and Lazar, 2004), was the only polymorphism demonstrated to alter type-2 diabetes susceptibility in a significant percentage of the general population (Altshuler et al., 2000). Recently however, variants with the gene encoding transcription factor 7-like 2 (TCF7L2) have been associated with a significant relative risk for the development of type-2 diabetes in a

large cohort comprised of Icelandic, Dutch, and American populations (Grant et al., 2006) although the functional relevance of these variants is currently unknown.

Recent studies suggest that the KCNJ11 gene, which encodes the Kir6.2 subunit of the K_{ATP} channel, is also a candidate diabetogenic gene. K_{ATP} channels are widely expressed throughout the body and are critical mediators of glucose homeostasis including proper GSIS from pancreatic β-cells. Recent clinical case studies have revealed a number of rare K_{ATP} channel mutations that contribute to the development of both transient and permanent neonatal diabetes (Yorifuji et al., 2005; Gloyn et al., 2004b). Molecular studies have shown that these mutations result in small increases in K_{ATP} channel activity as a result of decreased ATP sensitivity. While these mutations are rare, they highlight the important concept that even small increases in K_{ATP} channel activity can be sufficient to significantly impair insulin secretion.

Few more common genetic mutations (polymorphisms) in the K_{ATP} channel that may contribute to increased susceptibility to type-2 diabetes have been identified. However, the recently described E23K K_{ATP} channel polymorphism has received attention as it is found at a higher frequency in the Caucasian type-2 diabetic population (Nielsen et al., 2003) as well as at higher frequency in a population of Scandinavian women with gestational diabetes (Shaat et al., 2005). Indeed, E23K is among a very small group of variants found to significantly alter relative risk for type-2 diabetes that includes the PPARγ(Pro12Ala) mutation (Altshuler et al., 2000; Love-Gregory et al., 2003) and the newly discovered TCF7L2 variants (Grant et al., 2006). Furthermore, a potential association of the E23K Kir6.2 polymorphism with obesity (Nielsen et al., 2003), suggests a role for free fatty acids or their metabolites in the modulation of

polymorphic K_{ATP} channel activity and perhaps β-cell function. Recent studies have addressed this issue by examining the functional effects of these Kir6.2 polymorphisms on the K_{ATP} channel.

The Kir6.2 subunit forms both the potassium ion-conducting pore region and the ATP sensor of the K_{ATP} channel. Therefore, mutations that alter the ability of the channel to properly respond to ATP may lead to a decreased GSIS response and increased susceptibility to type-2 diabetes. Intensive research has been focused on three particular single nucleotide polymorphisms (E23K, L270V, and I337V) found in several Caucasian populations (Hani et al., 1998; Hansen et al., 1997; Inoue et al., 1997; Sakura et al., 1996). While L270V and I337V are found at similar frequency in both healthy and type-2 diabetic individuals, the E23K polymorphism appears at higher frequency in type-2 diabetic populations. The E23K and I337V polymorphisms are highly linked with reported concordance rates between 72% and 100% (Hani et al., 1998; Hansen et al., 1997; Sakura et al., 1996) depending on the population studied. In addition, recently published studies indicate that a number of Kir6.2 mutations including the activating R201H mutation (See chapter 6 for further discussion on the R201H mutation), may contribute to permanent neonatal diabetes (Gloyn et al., 2004b; Gloyn et al., 2004a; Sagen et al., 2004).

E23K is a missense single nucleotide polymorphism (gag→aag) located in the cytosolic proximal (5') N-terminal tail of the Kir6.2 subunit (Figure 1-5) that results in the substitution of a highly conserved glutamic acid (E) residue with lysine (K) and a subsequent negative-to-positive shift in residue charge. Although E23K is linked (>72%) with I337V (atc→gtc; no charge change), no discernable functional relevance of the

I337V polymorphism has been determined to date (Schwanstecher et al., 2002a; Schwanstecher et al., 2002b). However, the region surrounding the I337V polymorphism (residues 334 – 338) has been implicated in ATP-binding (Drain et al., 1998). In addition, the high concordance between E23K and I337V suggests that these polymorphisms may have originated in a common ancestor, pointing towards a possible evolutionary advantage to their maintenance in the general population.

Although initial studies using small cohorts of 150 to 350 subjects failed to detect association between E23K and type-2 diabetes ('t Hart et al., 2002; Hansen et al., 1997; Inoue et al., 1997; Sakura et al., 1996; Tschritter et al., 2002; Yamada et al., 2001), more recent larger studies have yielded an average genotypic frequency of 18% K/K for type-2 diabetic individuals versus 10.5% K/K in the glucose-tolerant general population (Table 1-1; Nielsen et al., 2003). This work is supported by two additional large-scale studies that independently confirm an association between E23K and type-2 diabetes (Florez et al., 2004; Gloyn et al., 2003), yielding odds ratios between 1.15 and 1.65 and further highlighting the importance of statistical power associated with large cohorts in genetic association studies.

While an epidemiological association has been made between E23K and type-2 diabetes, a mechanism that could account for the increased risk has not yet been determined. While initial studies examined the potential for altered ATP sensitivity in these mutants with conflicting results, my work indicates that a link may exist between elevated intracellular acyl CoA levels and impaired β-cell electrical excitability in the presence of the E23K polymorphism.

Section 2: Sodium/Calcium Exchanger Studies

Cardiac Substrate Metabolism and Electrophysiology

Proper functioning of individual cardiomyocytes relies on a combination of appropriate ionic homeostasis as accomplished through the concerted functioning of a number of ion channels and transporters, and appropriate substrate metabolism. The appropriate functioning of these two facets of cardiomyocyte activity culminate in the timely and coordinated contraction of individual cells that leads to the proper conduction of both electrical and contractile signals through the entire heart. Understandably, dysfunction of either substrate metabolism or ionic homeostasis can have deleterious consequences on the activity of the whole heart. This can manifest itself as contractile and electrical abnormalities which, depending on their severity, can prove fatal.

Cardiomyocytes derive energy in the form of ATP from the coordinated metabolism of two major substrates – glucose and fatty acids. Under normal working conditions, the heart preferentially derives ATP from the oxidation of fatty acids, while an additional smaller amount of energy is derived from glucose oxidation (for review, see Stanley et al., 2005). The rate at which fatty acids are taken up into cardiomyocytes is directly related to the plasma concentration of free fatty acids, which exists in the sub-millimolar range (~0.5 mM) in normal healthy individuals (Lopaschuk et al., 1994; Stanley et al., 2005). Once transported across the cell membrane, these fatty acids are normally esterified to CoA to form acyl CoAs that can then be stored as triglycerides, bound to a variety of other molecules including fatty acid and acyl CoA binding proteins or transported into the mitochondria where they serve as the substrate for β-oxidation. Under normal circumstances, free acyl CoA levels in the cytosol are highly buffered to

sub-micromolar concentrations by a number of fatty acid and acyl CoA binding proteins (Faergeman and Knudsen, 1997), therefore reducing the amount of free acyl CoA available to interact with ion channels and other proteins. However, under certain circumstances, including ischemia, obesity, and diabetes, plasma free fatty acid levels can increase to levels greater than 1 mM (Lopaschuk et al., 1994; Golay et al., 1986; Reaven et al., 1988). This increase in plasma fatty acids is accompanied by an increase in the cytosolic levels of both non-esterified fatty acids and acyl CoAs (Whitmer et al., 1978) and is particularly evident in obese and type-2 diabetic individuals (Sharma et al., 2004). Under normal aerobic conditions, the rate of β-oxidation can be increased to metabolize the additional fatty acids. Glucose oxidation is concomitantly reduced to maintain proper ATP generation. However, if this increase in fatty acids and acyl CoAs is accompanied by reduced oxygen consumption such as occurs during myocardial ischemia, reduced β-oxidation can lead to cytosolic acyl CoA accumulation (Whitmer et al., 1978) and the possibility of cardiac dysfunction resulting from acyl CoA-mediated modulation of ion channels and transporters.

The appropriate conduction of electrical impulses through cardiomyocytes is equally important for proper contractile function. A wide variety of ion channels, pumps and transporters are involved in maintaining appropriate excitation-contraction coupling. These include voltage-gated sodium and calcium channels, voltage-gated and inward-rectifying potassium channels, the sodium/potassium ATPase, the sodium/hydrogen exchanger and the sodium/calcium exchanger. Each of these proteins plays a different role in generating the cardiac action potential, calcium transients, and maintaining overall ionic balance. Depending on the expression level of the various channels, transporters

and pumps, different action potential waveforms can be generated in different areas of the heart. The reader is directed to the following excellent reviews detailing the expression and roles of ion channels in the many regions of the heart (Schram et al., 2002; Nerbonne and Kass, 2005). For the purposes of this thesis, only the sodium/calcium exchanger will be detailed here (see below) and examined experimentally (See chapter 4).

Normal cardiac substrate metabolism and electrical activity can be altered in a number of pathological settings including ischemia, hypertrophy, heart failure, obesity, and diabetes (Stanley et al., 2005; Rodrigues et al., 1998). Under these circumstances, changes in substrate supply, gene expression, and oxygen levels can lead to cardiac dysfunction and the generation of potentially fatal arrhythmias (Stanley et al., 1997). The setting of cardiac ischemia is particularly interesting and relevant in the context of the work presented in this thesis. As detailed above, aerobic/oxidative metabolism of acyl CoAs in the mitochondria serves as the major source for ATP production in the normal working heart. During ischemia, the availability of oxygen is reduced leading to a dramatic reduction in the oxidative capacity of the myocyte. This has several effects on both metabolism and electrical function of the cell. First, the reduction in acyl CoA β-oxidation can lead to an accumulation of free acyl CoAs in the cytosol (Whitmer et al., 1978). Secondly, the decrease in fatty acid oxidation can lead to a rise in the rate of glycolysis. Conversion of glucose to pyruvate produces excess protons, reducing the pH of the cytosol. To correct for this drop in pH, the sodium/hydrogen exchanger pumps protons out of the cell in exchange for sodium (Stanley et al., 1997). This attempt to stabilize pH however leads to intracellular sodium loading and the subsequent activation of reverse-mode sodium/calcium exchange. The end result is a global rise in intracellular

calcium levels that can then trigger arrhythmias and cell death (Vassalle and Lin, 2004). The fact that cardiac arrhythmias may be generated as a direct result of calcium loading via reverse-mode sodium/calcium exchange suggests that this protein may also be an important pharmacological target in the treatment of myocardial ischemia/reperfusion injury (Hobai and O'Rourke, 2004). It is therefore crucial to understand the molecular and functional aspects of this important ion exchanger.

The Sodium/Calcium Exchanger

Molecular Aspects of the Sodium/Calcium Exchanger

The sodium calcium exchangers (NCX) are a family of membrane-spanning proteins that are important for the regulation of calcium homeostasis in a number of cell types. The first NCX gene (NCX1) was cloned from canine heart tissue some 16 years ago (Nicoll et al., 1990). Subsequently, two additional gene products sharing approximately 70% sequence identity have been identified, encoding NCX2 and NCX3 (Li et al., 1994; Nicoll et al., 1996). While NCX1 expression is widespread, NCX2 and NCX3 expression appear limited to the brain and skeletal muscle (Li et al., 1994; Nicoll et al., 1996).

Initially NCX1 was thought to contain 12 membrane-spanning domains separated by a large intracellular loop (Nicoll et al., 1990). Although a crystal structure has yet to be elucidated, the current accepted molecular make-up of the NCX1 protein shows nine transmembrane domains, five of which are on the N-terminal side of the large intracellular loop and 4 of which are on the C-terminal side (Figure 1-6 and see Quednau et al., 2004). Many portions of the exchanger have been implicated in either the transport of ions across the membrane or modulation of NCX activity. The reader is directed to the

following excellent reviews on this topic (Shigekawa et al., 2002; Blaustein and Lederer, 1999). For purposes of this thesis, a small number of regulatory regions will be discussed in further detail.

The large intracellular loop located between transmembrane domains five and six contains a number of regulatory regions as well as a region of alternative splicing. Located just below the level of the membrane and spanning amino acids 251 – 270 is a region designated the e**x**changer **i**nhibitory **p**eptide (XIP; Li et al., 1991). Previous studies have shown that application of a synthetic peptide with this sequence potently inhibits exchanger activity. In addition, mutation studies indicate that this sequence is important in the secondary regulation of exchanger activity (Matsuoka et al., 1997 and see below for details). Although it is currently unclear how this peptide sequence regulates exchanger activity, studies have indicated that it may serve as a binding region for the anionic phospholipid, PIP_2 (He et al., 2000 and see chapter 4 for further details).

In addition to the XIP sequence, the large intracellular loop of NCX1 contains a region of alternative splicing that spans approximately 77 amino acids between residues 601 and 677 (Quednau et al., 1997). This region consists of a cassette of up to 5 exons ranging in size from 5 to 35 amino acids. NCX1 splice variants are created by combining different exons (labeled A through F). Several studies have examined the tissue specificity of NCX1 isoform expression (Lee et al., 1994; Quednau et al., 1997; Van Eylen et al., 2001). Currently 13 isoforms of NCX1 have been identified in tissues including but not limited to the brain, heart, lung, liver, kidney and pancreas (Quednau et al., 1997; Van Eylen et al., 2001). In the heart, where NCX activity was first demonstrated, expression is limited primarily to the NCX1.1 isoform (exons ACDEF;

Lee et al., 1994). Conversely, in pancreatic islets and several insulinoma cell lines, several isoforms have been identified including NCX1.3 (exons BD), NCX1.4 (exons AD), and NCX1.7 (exons BDF; Van Eylen et al., 2001). Different expression patterns will likely contribute to different overall NCX1 activity in these various tissues. Interestingly, the region of alternative splicing is significantly downstream of the XIP region as well as the internal regulatory calcium-binding region (Figure 1-6). Therefore, these regions are completely conserved across all splice variants. Indeed, at the amino acid level, the XIP region is identical not only within splice variants, but also across species (Table 1-2; K. Hamming, personal communication). The conservation of this sequence across isoforms and species may indicate its relative importance in the proper functioning of the exchanger.

Functional Aspects of the Sodium/Calcium Exchanger

NCX1 activity is regulated by both the concentration gradients for sodium and calcium as well as the driving force for those ions (i.e. the membrane potential). As these parameters are in constant flux in excitable cells such as cardiomyocytes or pancreatic β-cells, activity of NCX1 is constantly changing. Depending on the electrochemical gradient of the cell, the exchanger can operate in either calcium extrusion or calcium influx mode. These modes of operation are known as forward- and reverse-mode respectively (Figure 1-7). In either case, for each calcium ion that is transported across the membrane, 3 to 4 sodium ions are counter-transported producing a net flow of charge in the direction of sodium flow. Therefore, in forward-mode operation, an inward current is generated (Figure 1-7B), whereas during reverse-mode operation, an outward current is

produced (Figure 1-7C). These currents can be measured using standard patch clamp techniques.

The direction of current flow can be calculated theoretically using a modified Nernst equation (Blaustein and Lederer, 1999):

$$E_{Na/Ca} = n\ E_{Na} - 2\ E_{Ca} \quad (1\text{-}1)$$

where n is the coupling ratio of sodium ions to calcium ions (i.e. the stoichiometry of sodium/calcium exchange) and E_{Na} and E_{Ca} are the reversal potentials for the respective ion represented by the equations:

$$E_{Na} = RT/zF \times \ln([Na^+]_o/[Na^+]_i) \quad (1\text{-}2)$$

$$E_{Ca} = RT/zF \times \ln([Ca^{2+}]_o/[Ca^{2+}]_i) \quad (1\text{-}3)$$

where R is the gas constant, T is the temperature in Kelvin, z is the ionic valence (1 for Na^+, 2 for Ca^{2+}), and F is the Faraday constant.

While experimental data confirming the validity of equation 1-1 are somewhat lacking, we can speculate on the physiological roles of NCX1.1 in cardiac tissue and NCX1.3 in β-cells based on experimentally derived ion concentrations and membrane potentials. It is important to note that the exact value of n in equation 1-1 is unknown. Although most studies suggest a coupling ratio of 3 Na^+ ions for 1 Ca^{2+} ion (Hinata and Kimura, 2004), more recent studies show that n is variable within a given system and can possess values ranging between 3 and 5 (Fujioka et al., 2000). Hilgemann's group recently determined the optimal average coupling ratio for sodium/calcium exchange to be 3.2 (Kang and Hilgemann, 2004).

Cardiac NCX

Under normal circumstances, extracellular ion concentrations are relatively stable with $[Na^+]_o$ at ~ 140 mM and $[Ca^{2+}]_o$ at ~ 1.5 mM. In a typical cardiomyocyte, $[Na^+]_i$ can range from 4 to 14 mM while $[Ca^{2+}]_i$ can range from 100 nM at rest to as high as 1 μM during systole (Despa et al., 2002; Shattock and Bers, 1989; Gray et al., 2001; Bers and Guo, 2005). Using these concentrations and a coupling ratio of 3.2, we can calculate $E_{Na/Ca}$ during diastole to lie between -60 and +40 mV while during systole, $E_{Na/Ca}$ is positive at all $[Na^+]_i$. With the diastolic membrane potential sitting in the range of -80 mV, the exchanger should theoretically operate in forward mode to extrude calcium from the cell. Experimentally, it has been shown that forward-mode NCX1.1 activity contributes to the plateau phase of the action potential via the depolarizing influence of sodium influx (Janvier and Boyett, 1996).

Conversely, at the peak of the action potential, the membrane can be significantly depolarized to potentials as high a +60 mV. Under these circumstances, the exchanger can reverse operation, bringing calcium into the cell in exchange for sodium. There is some evidence to suggest that this brief reversal of NCX activity may contribute to the initiation of contraction via calcium-induced calcium release (Levi et al., 1993). However, it is likely that reverse-mode NCX1.1 activity plays a predominantly pathophysiological role in mediating calcium overload under conditions of elevated intracellular sodium including ischemia as detailed above.

Figure 1-8A shows the activation of forward and reverse-mode cardiac NCX1.1 activity. As previously described (Hilgemann et al., 1992; Matsuoka et al., 1995; Hilgemann, 1990), this isoform generates a non-inactivating current in forward-mode but

exhibits a slowly developing inactivation during reverse-mode operation. Many studies have examined the molecular mechanisms underlying this reverse-mode inactivation. Initial studies revealed that inactivation likely occurs when the intracellular sodium binding sites are fully loaded and thus this type of inactivation has also been termed sodium-dependent (or I_1) inactivation (Hilgemann et al., 1992). More recent studies also implicate the XIP region in mediating I_1 inactivation. Indeed, point and deletion mutations within this region can enhance or abolish I_1 inactivation (Matsuoka et al., 1997). The molecular mechanisms underlying the sodium- and XIP-dependent inactivation process remain to be fully characterized.

β-Cell NCX

The role of NCX activity in the β-cell is less clear. Only recently have studies begun to elucidate a physiological role for this protein in regulating insulin secretion. However, using equation 1-1, we can infer the physiological modes of operation in a resting and active β-cell. While we assume the external ion concentrations are the same as above, experimental evidence suggests that $[Na^+]_i$ decreases from 14 mM at rest to 11 mM when stimulated with glucose (see Saha and Grapengiesser, 1995 and chapter 5 for more details). Meanwhile, $[Ca^{2+}]_i$ sits at ~100 nM in a resting cell and can reach levels as high as 1 μM during the peak of the calcium-driven action potential (Gall et al., 1999; Van Eylen et al., 1998). Therefore, and again using a coupling ratio of 3.2, we can establish a theoretical $E_{Na/Ca}$ of -57 mV for a resting β-cell and an $E_{Na/Ca}$ of +20 mV for an active β-cell. As before, an examination of typical membrane potentials yields important information about the likely mode of operation during β-cell activity. At low

extracellular glucose concentrations (<5 mM), β-cells possess a resting membrane potential in the range of -60 to -70 mV. This membrane potential progressively depolarizes as glucose levels rise, until the threshold potential for activation of the voltage-gated calcium channels is reached. At the peak of the calcium-driven action potential, the membrane can reach potentials near but usually slightly below 0 mV (Henquin and Meissner, 1984). Therefore, either at low or high glucose levels, the β-cell membrane potential is consistently more negative than $E_{Na/Ca}$. Thus, NCX should operate exclusively in forward-mode. In cells that accumulate less calcium during stimulation, $E_{Na/Ca}$ can be calculated in the range of -10 to 0 mV (assuming a $[Ca^{2+}]_i$ of 300 – 400 nM). Under these conditions, it is theoretically possible for the exchanger to briefly flip into reverse-mode if the membrane potential were to become more positive than these values. There is evidence showing that artificial depolarization of β-cells using 50 mM K^+ can invoke reverse mode NCX activity (Van Eylen et al., 1998) but it has been suggested that reverse-mode NCX activity in β-cells is unlikely to contribute significantly to increasing intracellular calcium levels (Garcia-Barrado et al., 1996). The physiological role of this protein in regulating either membrane potential or intracellular calcium homeostasis remains controversial.

Similar to NCX1.1, the major β-cell isoform of the exchanger (NCX1.3) is subjected to an inactivation process when operating in the reverse-mode (Figure 1-8B). However, unlike NCX1.1, a slowly developing inactivation also occurs when NCX1.3 operates in forward-mode. The molecular mechanisms that mediate this forward-mode inactivation process are not currently known. As mentioned above, NCX1.3 and NCX1.1

share substantial sequence identity and known regulatory regions are conserved in these two isoforms. Therefore, the differences in observed inactivation properties between NCX1.1 and NCX1.3 must lie either directly within the alternative splicing region or the mechanism by which this region interacts with the folded protein.

Specific Aims of this Thesis

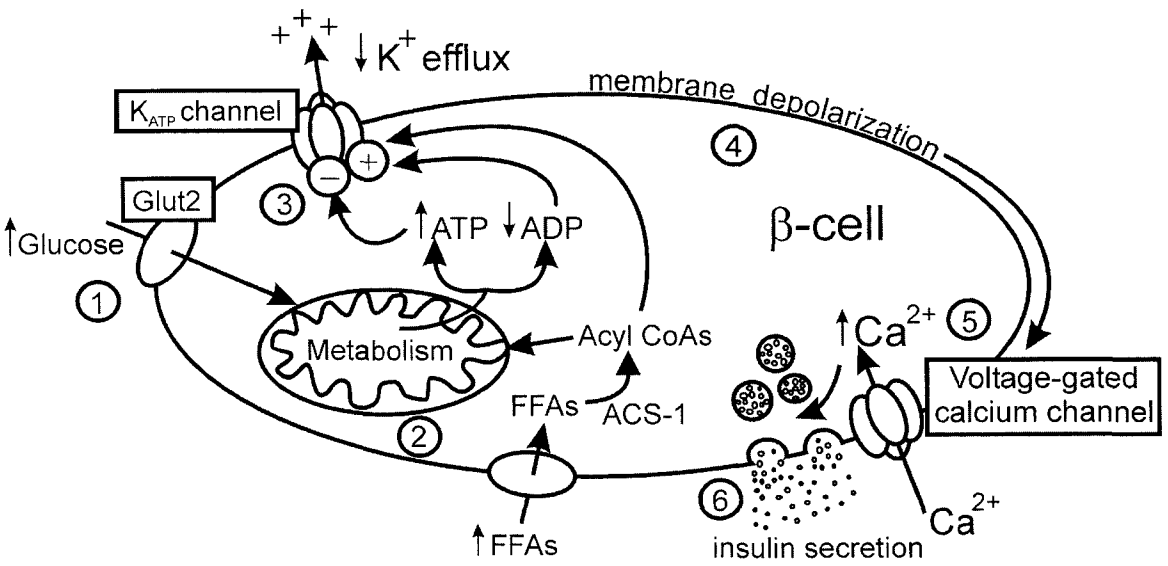
The initial objective of the research detailed in this thesis was to characterize the effects of long chain fatty acyl CoA esters on K_{ATP} channel activity with the primary goal of providing further insight into the molecular mechanisms that underlie the documented increase in risk for the development of type-2 diabetes in individuals with elevated levels of circulating lipids. The natural progression of my earlier work has led to the identification of a novel target of acyl CoA modulation, the sodium/calcium exchanger. A multidisciplinary approach using both molecular and electrophysiological techniques has allowed me to characterize the effects of long chain acyl CoAs on both the K_{ATP} channel and NCX, providing novel information that has direct relevance for both type-2 diabetes and cardiac ischemia/reperfusion injury.

Long chain acyl CoAs are important intracellular molecules, being used for both energy production and as signaling molecules for a variety of processes. The composition and concentration of intracellular acyl CoAs are highly dependent on dietary fat intake and energy balance, especially in 'Western' societies where there tends to be over-consumption of high caloric, high fat foods and a relative absence of regular physical activity. The composition of dietary fat has been linked to increased risk for the development of a number of pathologies including obesity, type-2 diabetes, coronary heart disease and myocardial infarction. In particular, risk for the development of these

diseases is associated with the consumption of saturated and *trans* fatty acids (Meyer et al., 2001; Salmeron et al., 2001). Conversely, diets higher in polyunsaturated fatty acids, including the fish oils docosahexaenoic acid (DHA) and eicosapentaenioc acid (EPA) have been linked to a reduction in risk for the above mentioned diseases (Salmeron et al., 2001; De Caterina et al., 2006). It is noteworthy that current 'western' diets tend to be higher in both saturated and processed/*trans* fats than in these polyunsaturated fatty acids. While these previous epidemiological observations do not examine molecular mechanisms and it is a major aim of this study to identify any relationship that may exist between dietary fat composition and modulation of either K_{ATP} channels or the sodium/calcium exchanger.

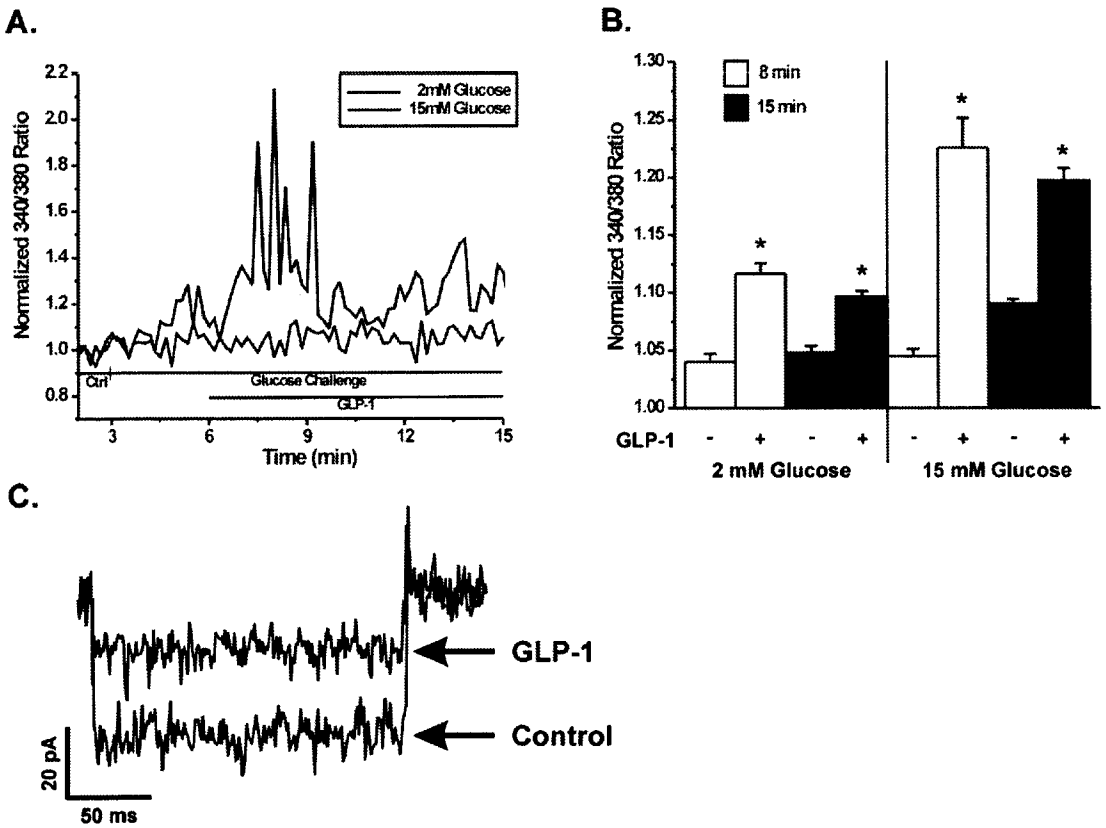
Finally, a link between the E23K Kir6.2 polymorphism and type-2 diabetes has now been firmly established. However, the molecular mechanisms underlying this increased disease susceptibility have not been elucidated. Although recent evidence suggests that this polymorphism decreases ATP sensitivity of the K_{ATP} channel, we report an additional sensitization of K_{ATP} channels to activation by acyl CoAs, providing a potential molecular link between obesity, type-2 diabetes, and the E23K polymorphism.

Figure 1-1.



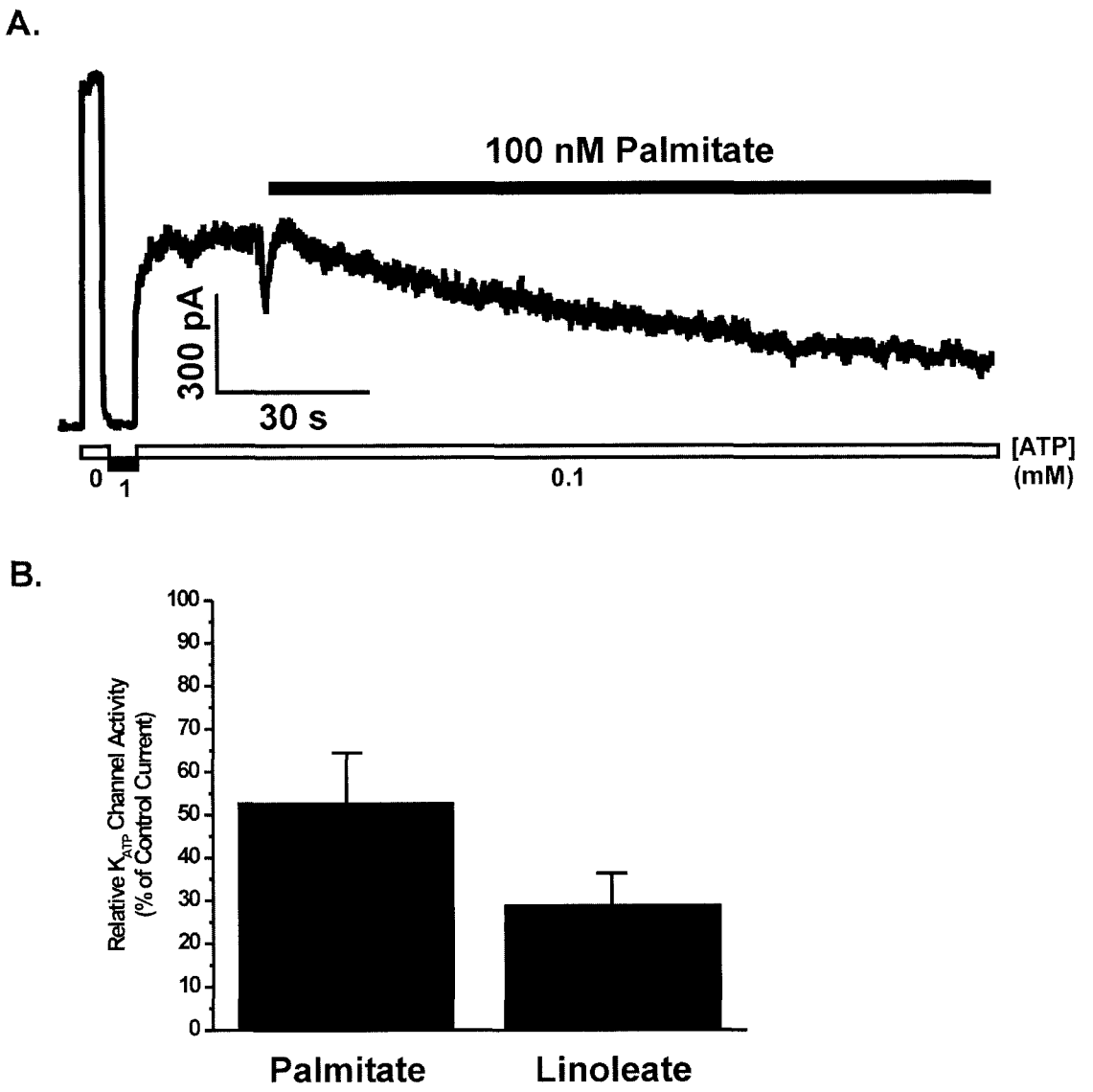
K_{ATP} channel-dependent mechanism of glucose-stimulated insulin secretion. ***1*** Glucose is transported into the pancreatic β-cell via the GLUT2 transporter. ***2*** Glucose oxidation in the mitochondria leads to an increase in the cytosolic ATP-to-ADP ratio, which inhibits the activity of K_{ATP} channels (***3***). ***4*** The reduction in K^+ efflux causes membrane depolarization and activation of voltage-gated (L-type) calcium channels (***5***). ***6*** Transient increases in cytosolic Ca^{2+} concentrations trigger the release of insulin-containing granules. ATP production can also arise via the activation of FFAs by ACS-1 and subsequent β-oxidation of LC-CoAs within the mitochondria. Additional ion channels are involved in the repolarization of the membrane potential and cessation of insulin release. Abbreviations: Glut2, glucose transporter isoform 2; FFAs, free fatty acids; ACS-1, acyl-CoA synthetase isoform 1; acyl CoAs, long-chain acyl CoA esters; ATP, adenosine triphosphate; ADP, adenosine diphosphate.

Figure 1-2.



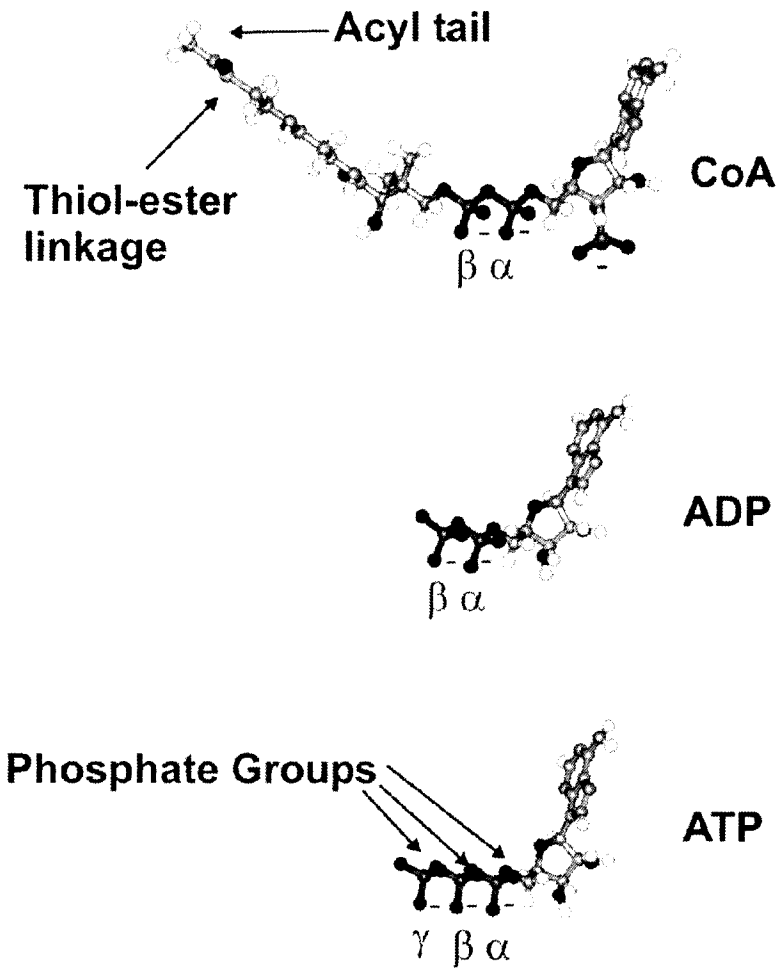
GLP-1 stimulates β-cell activity in a glucose-dependent manner. ***A.*** Measurements of intracellular calcium using the ratiometric fluorescent calcium indicator FURA-2 in the INS-1 β-cell line. While 2 mM glucose has little effect on intracellular calcium levels in the absence or presence of 20 nM GLP-1 (black line), 15 mM glucose induces a significant rise in calcium that is increased dramatically in the presence of GLP-1. ***B.*** Grouped data indicating that the effect of GLP-1 on increasing intracellular calcium is more than doubled in the presence of elevated glucose. $*P<0.05$ vs. respective glucose and time point in the absence of GLP-1. ***C.*** The effects of GLP-1 on increased calcium can be attributed to a decrease in the activity of K_{ATP} channels as measured in INS-1 cells using the perforated whole-cell patch-clamp technique in voltage-clamp mode. Panel C was adapted from (MacDonald et al., 2002) and is reprinted here with permission from The American Diabetes Association.

Figure 1-3.



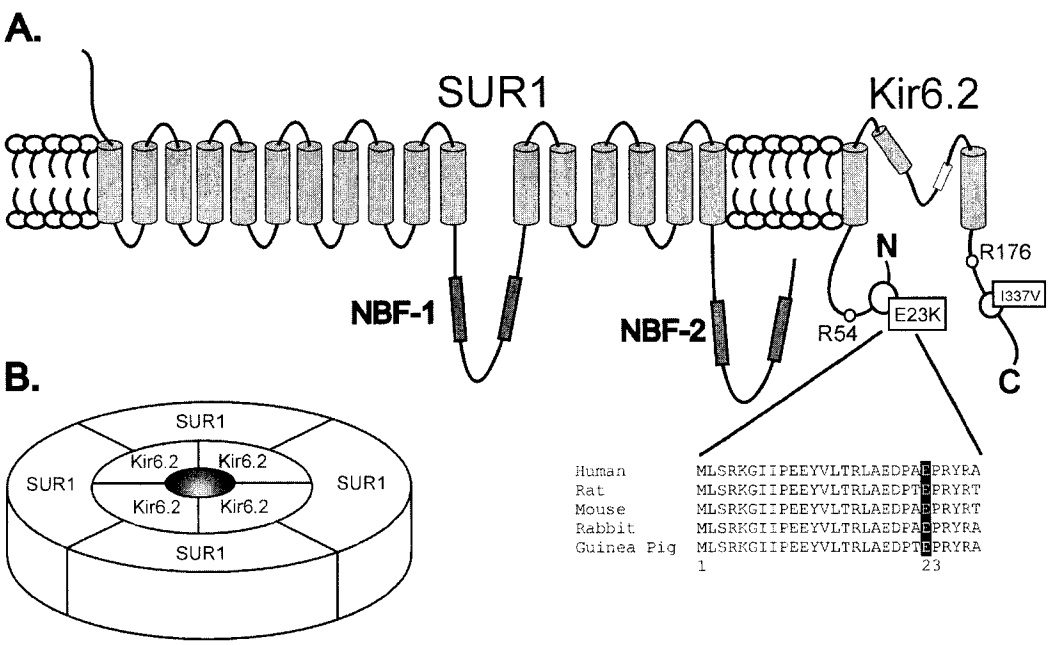
Free fatty acids inhibit K_{ATP} channel activity. ***A.*** Representative macroscopic K_{ATP} channel current recording from tsA201 cells transiently expressing recombinant K_{ATP} channels indicating that palmitate (100 nM) potently inhibits K_{ATP} channel activity. ***B.*** Grouped data showing that both saturated (palmitate; C16:0) and unsaturated (linoleate; C18:2) fatty acids inhibit K_{ATP} channels. There is a non-significant difference between these two fatty acids.

Figure 1-4.



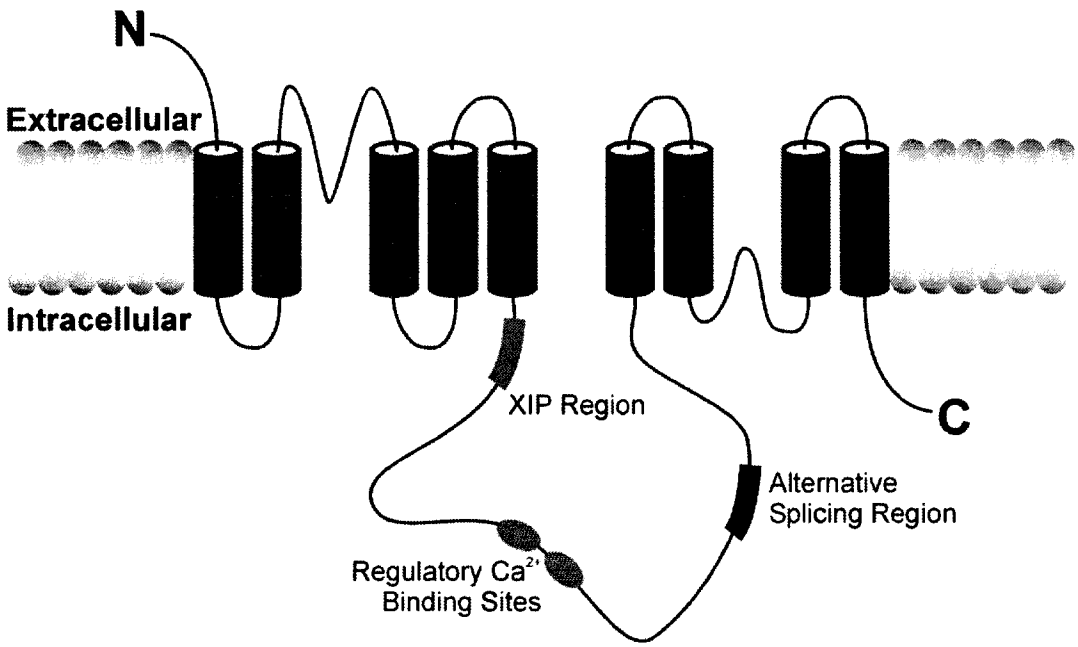
Schematic representation of molecular structures of ATP, ADP, and acyl CoAs. Note the similarities in the structure of the head group including the locations of the α and β phosphate groups. The sulfur atom responsible for the thiol-ester linkage between CoA and the attaching fatty acid is also highlighted.

Figure 1-5.



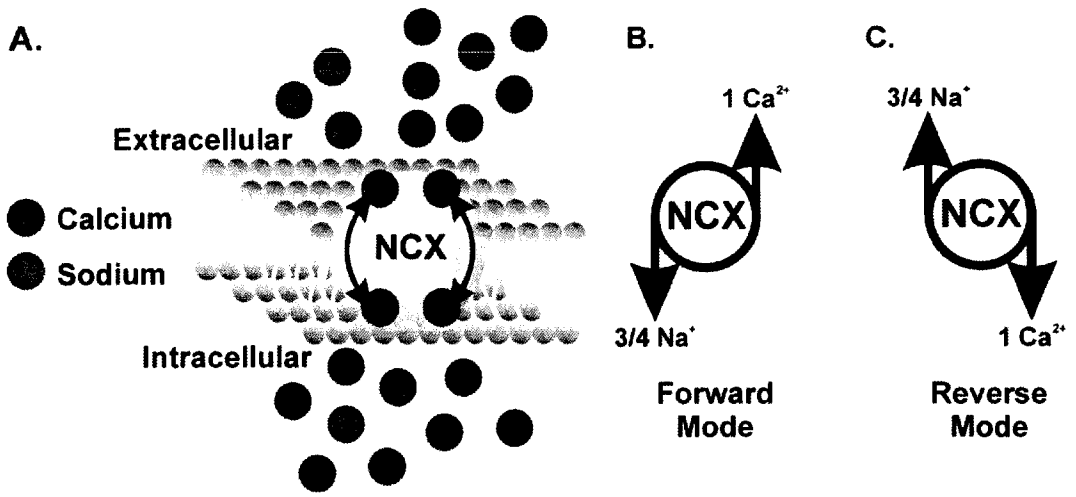
A. Schematic representation of the K_{ATP} channel subunits SUR1 and Kir6.2. The relative position of several important residues, including the E23K polymorphism in the proximal N-terminus if the Kir6.2 subunit, are highlighted. The region of E23K is expanded to highlight the inter-species conservation of this glutamic acid residue. ***B.*** The K_{ATP} channel is a hetero-octomeric complex comprised of four Kir6.2 subunits that together form the K^+-specific pore, surrounded by four SUR1 subunits.

Figure 1-6.



A schematic representation of the membrane topology of the sodium/calcium exchanger. Labeled are regions of interest for this particular study. The XIP region spans amino acids 251 – 270 and lies just below the plane of the plasma membrane. Two regulatory calcium binding sites have been located between residues 446 – 454 and residues 498 – 509 (Levitsky, 1994). Finally, the area spanning amino acids 601 – 677 represents the area of alternative splicing. Each of these regions is located on the large intracellular loop and their relative positions are indicated as shown.

Figure 1-7.



The sodium-calcium exchanger can operate to extrude or increase intracellular calcium. ***A.*** A schematic representation of the sodium-calcium exchanger embedded in the plasma membrane. Representation of the direction and stoichiometry of ion flow during forward-mode (***B***) and reverse-mode (***C***) sodium-calcium exchanger operation.

Representative macroscopic sodium-calcium exchange currents measured in both forward- and reverse-mode. ***A.*** Adenoviral-mediated infection of tsA201 cells with the cardiac isoform (NCX1.1) results in >75% infection efficiency (***Ai***). The resulting macroscopic current exhibits characteristic non-inactivating forward-mode activation (***Aiii***) whereas in reverse-mode, the current is subjected to I_1 inactivation (***Aii***). ***B.*** Co-transfection of the NCX1.3 plasmid with pGreenLatern (which encodes green fluorescent protein) results in >50% transfection efficiency (***Bi***). The β-cell isoform (NCX1.3) exhibits a similar inactivation process during reverse-mode operation (***Bii***) but in contrast to NCX1.1, also exhibits a slowly developing inactivation when operating in forward-mode (***Biii***). Currents were recorded using a modified excised inside-out patch-clamp technique as detailed in the materials and methods sections of chapters 4 and 5.

Figure 1-8.

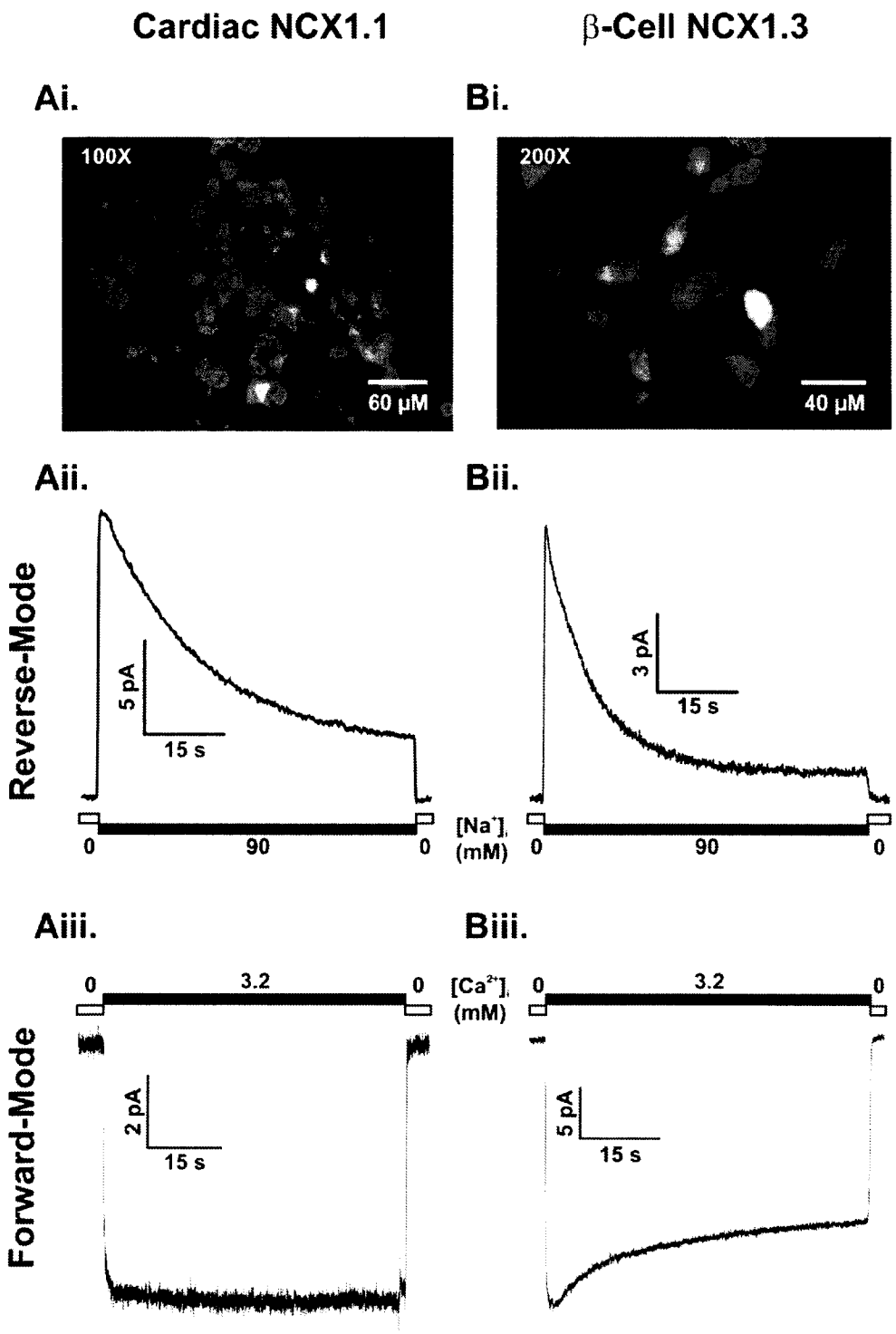


Table 1-1.

Table 1. Genotype frequencies of the Kir6.2 E23K polymorphism in all population studies performed to date.

	% Glucose-tolerant cohort (n)			% Type 2 diabetic cohort (n)			Reference
	E/E	E/K	K/K	E/E	E/K	K/K	
	0.54 (44)	0.33 (27)	0.13 (11)	0.38 (38)	0.45 (45)	0.17 (17)	Sakura *et al.*, 1996
	0.36 (59)	0.59 (96)	0.05 (9)	0.43 (124)	0.46 (133)	0.12 (34)	Inoue *et al.*, 1997
	0.40 (45)	0.46 (53)	0.14 (16)	0.28 (53)	0.45 (87)	0.27 (51)	Hani *et al.*, 1998
	0.41 (125)	0.50 (152)	0.09 (30)	0.37 (133)	0.45 (161)	0.18 (66)	Gloyn *et al.*, 2001
	0.40 (26)	0.49 (32)	0.11 (7)	0.36 (34)	0.53 (50)	0.11 (10)	't Hart *et al.*, 2002
	0.42 (491)	0.45 (534)	0.13 (157)	0.36 (308)	0.48 (412)	0.16 (134)	Gloyn *et al.*, 2003
	0.39 (330)	0.47 (408)	0.14 (124)	0.36 (287)	0.47 (382)	0.17 (134)	Nielsen *et al.*, 2003
Average (total n)	0.42 (1120)	0.47 (1302)	0.11 (354)	0.36 (977)	0.47 (1270)	0.17 (446)	
	0.436 (160)	0.452 (166)	0.112 (41)	0.353 (184)	0.453 (236)	0.194 (101)	Hani *et al.*, 1998 *
	0.378 (521)	0.518 (657)	0.105 (173)	0.363 (525)	0.458 (685)	0.18 (263)	Nielsen *et al.*, 2003 **

Data are fractions of each group with indicated genotype (number of subjects in group).
*Recently performed meta-analysis
**Averaged data from individual population studies by Nielsen *et al., 2003*, Gloyn *et al., 2001*, Hani *et al., 1997*, and Inoue *et al., 1997*.

Table 1-2.

Species	XIP Region Sequence																			
Human	AGG	AGA	CTT	CTG	TTT	TAC	AAG	TAT	GTC	TAC	AAG	AGG	TAT	CGA	GCT	GGC	AAG	CAG	AGG	GGG
	R	R	L	L	F	Y	K	Y	V	Y	K	R	Y	R	A	G	K	Q	R	G
Rat	AGG	CGG	CTT	CTC	TTT	TAC	AAG	TAC	GTC	TAC	AAG	CGG	TAC	AGG	GCT	GGC	AAG	CAG	AGG	GGG
	R	R	L	L	F	Y	K	Y	V	Y	K	R	Y	R	A	G	K	Q	R	G
Mouse	AGG	CGG	CTT	CTC	TTT	TAC	AAG	TAT	GTC	TAC	AAG	CGG	TAC	AGG	GCC	GGC	AAG	CAG	AGG	GGG
	R	R	L	L	F	Y	K	Y	V	Y	K	R	Y	R	A	G	K	Q	R	G

Sequence comparison of the XIP region of human, rat, and mouse species. Although slight differences exist at the single nucleotide level, the amino acid sequence is conserved in all species.

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Chapter 2

Kir6.2 Polymorphisms Sensitize β-cell ATP-sensitive Potassium Channels to Activation by Acyl CoAs: A Possible Cellular Mechanism for Increased Susceptibility to Type-2 Diabetes?

Previously published in *Diabetes* under the following citation:

Riedel,M.J., Boora,P., Steckley,D., de Vries,G., and Light,P.E. (2003). Kir6.2 Polymorphisms Sensitize β-cell ATP-sensitive Potassium Channels to Activation by Acyl CoAs: A Possible Cellular Mechanism for Increased Susceptibility to Type-2 Diabetes? Diabetes *52*, 2630-2635.

Introduction

Type-2 diabetes is a multi-factorial disease with both genetic and environmental components contributing to its development. Despite the investigation of polymorphic variations in genes encoding for key components in pathways controlling insulin secretion, their precise roles in the etiology of type-2 diabetes are not well understood.

Glucose homeostasis is maintained through the coordinated release of several hormones including insulin, glucagon, and glucagon-like peptide-1 (GLP-1). A key component regulating the release of these hormones is the ATP-sensitive potassium (K_{ATP}) channels (Ashcroft and Rorsman, 1989; Ashcroft, 2000; Reimann and Gribble, 2002). Hormone secretion in the pancreatic β- and α-cell, and in the intestinal L-cell is controlled through metabolic regulation of electrical activity, a process critically linked to glucose and fatty acid metabolism, which in turn regulates the activity of K_{ATP} channels that control membrane potential (Ashcroft and Rorsman, 1989; Ashcroft, 2000; Reimann and Gribble, 2002; Light, 2002).

The K_{ATP} channel is a hetero-octomeric protein comprised of the pore-forming inward-rectifier Kir6.2 subunit coupled to the high affinity sulphonylurea receptor SUR1 subunit (Inagaki et al., 1995; Aguilar-Bryan et al., 1995) in a stoichiometry of $(Kir6.2)_4/(SUR1)_4$. Mutations that reduce K_{ATP} channel activity can lead to the increased β-cell excitability and excessive insulin secretion that underlies the congenital disorder of persistent hyperinsulinemic hypoglycemia of infancy (PHHI; Aguilar-Bryan and Bryan, 1999). Conversely, several rare activating mutations have been identified in the K_{ATP} channel that reduce ATP sensitivity and lead to transient or permanent forms of neonatal diabetes (Gloyn et al., 2005; Yorifuji et al., 2005; Gloyn et al., 2004). In this respect, it

has also been demonstrated that targeted over-activity of K_{ATP} channels in certain transgenic animal models impairs insulin secretion and leads to a severe neonatal diabetic phenotype (Koster et al., 2000). Therefore, enhanced β-cell K_{ATP} channel activity reduces the magnitude of the glucose stimulated insulin secretion response in β-cells and can therefore lead to the development of diabetes.

A recently discovered set of three commonly occurring single nucleotide polymorphisms (E23K, L270V, and I337V) in the Caucasian population has been found within the Kir6.2 gene (Hani et al., 1998; Gloyn et al., 2001). The E23K and I337V polymorphisms appear to be tightly linked, with >72% of those possessing E23K also possessing I337V (Hani et al., 1998; Sakura et al., 1996). Recent clinical studies have indicated that homozygous polymorphic individuals are at an increased risk of developing type-2 diabetes compared to individuals with either heterozygous polymorphic or wild-type K_{ATP} channels (Hani et al., 1998; Sakura et al., 1996; Inoue et al., 1997; Hansen et al., 1997). Homozygous subjects also possess impaired glucose-stimulated insulin secretion (upon oral glucose challenge). The prevalence of the E23K Kir6.2 polymorphism may be linked to obesity as indicated by an increase in body mass index (BMI) among homozygous polymorphic individuals (Nielsen et al., 2003). In addition, recent evidence suggests that the E23K polymorphism may result in increased glucagon secretion during hyperglycemia (Tschritter et al., 2002) which could result from increased K_{ATP} channel activity (Gopel et al., 2000; Ashcroft, 2000). Taken together, the available evidence suggests the homozygous polymorphic population may be either especially susceptible to impairment of glucose homeostasis if they are overweight or obese and/or that these polymorphisms may predispose patients to diabetes and excessive

weight gain. However the cellular mechanisms underlying these associations are still poorly understood.

At the cellular mechanistic level, it has been shown that the E23K polymorphism can decrease ATP sensitivity, rendering K_{ATP} channels slightly more active at higher ATP concentrations (Schwanstecher et al., 2002). In addition, it is known that plasma free fatty acid (FFAs) levels are elevated in obese (Golay et al., 1986) and diabetic (Reaven et al., 1988) individuals with chronic elevation leading to an accumulation of long chain acyl CoAs (acyl CoAs) in the cytosol, which can then directly activate β-cell K_{ATP} channels (Branstrom et al., 1998; Larsson et al., 1996). Due to the cytosolic location of the polymorphisms in the Kir6.2 gene (Figure 2-2D) and their possible effects on ATP and acyl CoA sensitivity, we hypothesized that binding of acyl CoAs may further potentiate polymorphic K_{ATP} channel activity relative to wild-type channels, thus reducing cellular excitability and contributing to impaired nutrient-stimulated hormone secretion, such as reduced insulin secretion that is observed in homozygous polymorphic individuals with type-2 diabetes (Nielsen et al., 2003).

Results

Kir6.2(E23K/I337V) polymorphic K_{ATP} channels are more susceptible to concentration-dependent increases in activity by palmitoyl CoA.

Due to the coupling of the E23K and I337V polymorphisms *in vivo*, we have examined the sensitivity of the E23K/I337V double polymorphic K_{ATP} channel to activation by the common saturated long chain acyl CoA – palmitoyl CoA relative to the wild-type (Kir6.2/SUR1) K_{ATP} channel in a recombinant expression system. Application of palmitoyl CoA resulted in a concentration-dependent increase in current of both wild-

type (WT) and polymorphic K_{ATP} channels (Figure 2-1). At palmitoyl CoA concentrations of 100 nM and greater, polymorphic K_{ATP} channel current was significantly increased ~2-fold over WT at the same concentration (Figure 2-1C). For example, K_{ATP} channel current was increased by 389.69 ± 48.62% compared to 196.82 ± 20.30% in WT channels (1.98-fold increase, $P<0.05$) upon application of 100 nM palmitoyl CoA.

Contribution of the SUR1 subunit to the observed palmitoyl CoA-mediated increase in K_{ATP} channel activity.

To investigate the mechanisms responsible for the observed increase in sensitivity to acyl CoAs in the polymorphic K_{ATP} channel population, we utilized the Kir6.2ΔC26 truncation mutant to assess the contribution of the SUR1 subunit (Figure 2-2D). The Kir6.2ΔC26 mutant can be expressed to yield functional ATP-sensitive potassium currents in the absence of the accessory SUR1 subunit (Tucker et al., 1997). Although higher concentrations of palmitoyl CoA were required to activate the Kir6.2ΔC26 channel, a significant 1.52-fold increase in current magnitude in response to 500 nM palmitoyl CoA was observed in polymorphic Kir6.2ΔC26 channels (219.67 ± 37.27%) vs. WT (144.80 ± 17.20%) Kir6.2ΔC26 channels. This magnitude of increase was similar to that noted in K_{ATP} channels comprised of both the Kir6.2 and SUR1 subunits (789.91 ± 89.30% in polymorphic K_{ATP} channels vs. 355.80 ± 52.56% in WT K_{ATP} channels) where a 2.2-fold increase in activity was observed in the E23K/I337V polymorphic K_{ATP} channel population in response to 500 nM palmitoyl CoA (Figure 2-1C).

Polymorphic K_{ATP} channels exhibit greater open probability vs. wild-type channels in the presence of palmitoyl CoA.

Increases in open probability and/or single channel amplitude could result in the observed increase in macroscopic polymorphic K_{ATP} channel activity in response to palmitoyl CoA. In order to test which of these parameters is altered, we analyzed single channel openings of recombinant K_{ATP} channels expressed in tsA201 cells. Our results indicate that in the presence of 1 mM ATP, application of 100 nM palmitoyl CoA to wild-type or polymorphic K_{ATP} channels does not significantly alter single channel amplitude (Figure 2-3). At a holding potential of –60mV, wild-type channels displayed a single channel conductance of 60.24 ± 0.97 pS versus polymorphic channels (56.67 ± 2.70 pS; NS, n=7) suggesting that the observed increase in K_{ATP} channel activity occurs through changes in open probability.

It has previously been reported that the E23K polymorphism results in an increased spontaneous K_{ATP} channel open probability in the absence of ATP (Schwanstecher et al., 2002). In the present study, we examined the single channel properties of the E23K/I337V double polymorphism in tsA201 cells. In this recombinant expression system, we observed no significant difference between the spontaneous open probability ($P_{o,zero}$) of wild-type versus polymorphic K_{ATP} channels (WT: 0.837 ± 0.08 vs. E23K/I337V: 0.829 ± 0.04; NS, n=7) as measured by noise analysis in the absence of ATP.

ATP sensitivity in altered in the presence of palmitoyl CoA.

To gain insights into the physiological significance of these findings, we have examined ATP sensitivity in wild-type and polymorphic K_{ATP} channels in the absence

and presence of palmitoyl CoA (Figure 2-4). 100 nM palmitoyl CoA decreased ATP sensitivity in both wild-type and polymorphic K_{ATP} channel populations (Figure 2-4A), shifting the IC_{50} to the right 1.88-fold and 1.90-fold respectively. However, our results indicate that the ATP sensitivity of E23K/I337V polymorphic K_{ATP} channels is slightly increased at the respective IC_{50} concentrations versus wild-type channels both in the absence and presence of palmitoyl CoA (Figure 2-4B). Therefore, at low sub-millimolar ATP concentrations, the E23K/I337V polymorphic K_{ATP} channels were more likely to be closed than wild-type channels.

As physiological ATP concentrations in resting β-cells are estimated to be in the low millimolar range (Kennedy et al., 1999; Gribble et al., 2000), we examined this portion of the concentration-response curve in greater detail (Figure 2-4C). At 1 mM ATP, in the presence of 100 nM palmitoyl CoA, the polymorphic K_{ATP} channel population exhibited a significant increase in current (6.42 ± 1.21% of maximal K_{ATP} current vs. 3.61 ± 0.40% in WT; Figure 2-4D). The difference in effect at low versus high ATP concentrations occurred due to a reduction in the Hill coefficient of ATP binding from 2.28 in wild-type to 1.58 in polymorphic K_{ATP} channels (Figure 2-4E). After application of palmitoyl CoA, the Hill coefficients were further reduced to 1.41 in the wild-type and 1.24 in the polymorphic K_{ATP} channel population.

The change in Hill coefficient produces a crossing of the wild-type and polymorphic K_{ATP} channel concentration-response curves. In the presence of palmitoyl CoA, this crossover occurs at a concentration between 100 and 500 μM ATP, suggesting that at physiological $[ATP]_i$ the E23K/I337V polymorphic K_{ATP} channels will be more active than wild-type K_{ATP} channels (Figure 2-4C). From the ATP-inhibition curves, it

was calculated that the residual K_{ATP} channel activity at 1 mM ATP was approximately 40% greater in polymorphic K_{ATP} channels compared to wild-type K_{ATP} channels (Figure 2-4D).

Mathematical Modeling of β-Cell Electrical Activity.

Using a mathematical model of β-cell electrical activity, we found that increases in K_{ATP} channel conductance on the order of ~25% are adequate to significantly reduce the bursting behaviour characteristic of glucose-stimulated β-cells (Rorsman, 1997), suggesting that the observed increase in K_{ATP} channel activity in this study may be sufficient to alter insulin secretion (Figure 2-5). Using an initial plateau fraction of 0.18 (see research design and methods) chosen to represent β-cell electrical activity at 5.5 mM glucose (Figure 2-5Ai), the introduction of a 20% increase in K_{ATP} channel conductance, representative of the lower end of increased channel activity in the polymorphic K_{ATP} channel population, completely abolished electrical activity (Figure 2-5Aii). At a higher initial plateau fraction of 0.85 (representative of ~22 mM glucose; Figure 2-5Bi), an increase in K_{ATP} channel conductance of 25% significantly reduced the bursting behaviour (Figure 2-5Bii) to a level representative of ~8 mM glucose (Ozawa and Sand, 1986). Taken together, the results and simulations indicate that physiological palmitoyl CoA concentrations could lead to an increased basal polymorphic K_{ATP} channel activity resulting in impaired glucose stimulated insulin secretion.

Discussion

The aim of this study was to examine the molecular effects of the Kir6.2(E23K/I337V) polymorphism on K_{ATP} channel activity. Given the previously observed association of the E23K polymorphism with type-2 diabetes and obesity (Gloyn

et al., 2003; Nielsen et al., 2003), we tested the hypothesis that long chain acyl CoAs, known lipid modulators of wild-type K_{ATP} channel function, may differentially regulate polymorphic K_{ATP} channel activity. Our results indicate that acyl CoAs preferentially increase polymorphic K_{ATP} channel activity via decreasing ATP sensitivity at high ATP levels typically found in pancreatic β-cells.

Mechanistic Insights

Intracellular free acyl CoA levels are typically highly buffered by fatty acid and acyl CoA binding proteins to sub-micromolar concentrations. With estimates of β-cell cytosolic free acyl CoA in the range of 0.5 – 1 μM and binding affinities for various acyl CoA-utilizing enzymes in the nanomolar to micromolar range (Deeney et al., 1992), our results show that in the presence of physiological concentrations of palmitoyl CoA, polymorphic K_{ATP} channels are significantly more active than wild-type K_{ATP} channels.

Both the E23K and I337V polymorphisms are located in the cytosolic portion of the Kir6.2 subunit suggesting that they may be accessible to intracellular modulators that act on this subunit. We tested the role of the SUR1 subunit in mediating the observed increase in K_{ATP} channel activity by expressing the Kir6.2ΔC26 mutant in tsA201 cells. This deletion mutation removes the C-terminal 26 amino acids that include the ER-retention signal normally masked by co-expression of the SUR1 subunit (Tucker et al., 1997). As a result, the Kir6.2 subunit can be expressed alone to form a functional K_{ATP} channel. We show here that although higher acyl CoA concentrations are required to elicit a response, the Kir6.2ΔC26 mutant remains responsive to palmitoyl CoA. In addition, E23K/I337V polymorphic channels are activated to a greater extent than wild-type channels. These results, together with the observation that sulphonylurea sensitivity

is unaffected in the presence of the E23K polymorphism (Schwanstecher and Schwanstecher, 2002), suggest that the principal mechanism by which the E23K/I337V polymorphism alters acyl CoA sensitivity lies primarily within the Kir6.2 subunit.

Previous studies have examined the effects of the E23K polymorphism on K_{ATP} channel ATP sensitivity. In particular Schwanstecher and colleagues have reported an increased spontaneous open probability in K_{ATP} channels containing the single Kir6.2 E23K polymorphism in the absence of ATP. In this same study they noted a rightward shift in the ATP concentration-inhibition curve at all tested ATP concentrations and suggested therefore that the effect of the E23K polymorphism was to decrease ATP sensitivity, rendering the K_{ATP} channel more active at any given ATP concentration (Schwanstecher et al., 2002). Our results however, do not confirm the observed increase in spontaneous open probability, noting no significant change in this parameter between wild-type and E23K/I337V polymorphic K_{ATP} channel populations. Instead, we found that the slope of the ATP concentration-response curve was altered in the E23K/I337V polymorphic K_{ATP} channel population both in the presence and absence of palmitoyl CoA. This change in slope resulted from a decrease in the Hill coefficient and produced an interesting result. At low ATP levels, E23K polymorphic channels were less active than wild-type K_{ATP} channels. However, at physiological intracellular ATP levels (above 1 mM), E23K/I337V polymorphic K_{ATP} channels were less likely to close. The reduction in Hill coefficient suggests that the ability of ATP to reach its binding site(s) may be impaired in the presence the E23K/I337V polymorphism. A further impairment of ATP binding after exposure to palmitoyl CoA may occur due to competition at the ATP/Acyl CoA binding site. A similar effect on ATP-inhibition has been noted in cardiac

sarcolemmal K_{ATP} channels in response to protein kinase C (Light et al., 2000). The exact molecular mechanism behind this shift in Hill coefficient is unknown however it may involve a slightly altered tertiary structure of the polymorphic K_{ATP} channel that renders it more closed at low ATP concentrations, but more susceptible to activation by acyl CoAs.

Physiological Implications

Obesity is a major risk factor for the development of type-2 diabetes. The association of the E23K polymorphism with increased BMI (Nielsen et al., 2003) suggests that a link may exist at the molecular level between lipids or lipid metabolites and altered insulin secretion. With K_{ATP} channels being a major determinant of the resting membrane potential of a β-cell and a crucial transducer of metabolic signals to changes in electrical excitability, we used mathematical modeling to assess the effects of altered K_{ATP} channel activity on the electrical activity of a modeled β-cell. Using the results obtained experimentally in this study, we found that even a slight increase in K_{ATP} channel activity (~25%) can have dramatic effects on the bursting behaviour of a modeled β-cell. The requirement for a larger depolarizing stimulus, such as increased glucose, prior to the initiation of insulin secretion under these circumstances has obvious physiological implications. Elevated plasma glucose levels are a prerequisite for the development of type-2 diabetes and given the effect of the E23K polymorphism on β-cell membrane potential, it is possible that in individuals with this polymorphism, the addition of a high fat diet or obesity could be the trigger for developing impaired insulin secretion.

Implications for Other Tissues

Glucose homeostasis is properly maintained through a balanced secretion of hormones from multiple tissues. Interestingly, the K_{ATP} channel is involved in appropriate secretion of insulin (Ashcroft and Rorsman, 1989), glucagon (Gopel et al., 2000), and GLP-1 (Reimann and Gribble, 2002; Reimer et al., 2001). Neuronal control of food intake/satiety and hormone secretion via glucose-sensing neurons in the hypothalamus represents an additional potentially impaired process in homozygous polymorphic individuals as K_{ATP} channels are highly expressed in various regions of the CNS including the hypothalamus (Miki et al., 2001). It is therefore plausible that in addition to impaired insulin secretion from β-cells, the E23K/I337V polymorphism may result in dysfunctional secretion of glucagon and GLP-1 as well as impaired regulation of food intake.

Summary

In summary, we propose that increased polymorphic K_{ATP} channel activity in the presence of normal or elevated levels of intracellular acyl CoAs may result in decreased insulin and GLP-1 secretion coupled with increased glucagon release. Together, the dysfunctional secretion of these hormones may contribute to the association of the E23K/I337V Kir6.2 polymorphisms with type-2 diabetes. Additional studies will be required to directly assess altered hormone secretion in cell lines expressing a homozygous population of polymorphic K_{ATP} channels. This study is the first to propose a mechanistic link between increased BMI and plasma free fatty acid levels and the observed high prevalence of type-2 diabetes in the K_{ATP} channel homozygous polymorphic population.

Research Design and Methods

Cell Culture and Transfection. tsA201 cells (a SV40-transformed variant of the HEK293 human embryonic kidney cell line) were maintained in DMEM supplemented with 25 mM glucose, 2 mM L-glutamine, 10% fetal calf serum, and 0.1% penicillin/streptomycin. Cells were kept at 37°C with 5% CO_2. The K_{ATP} channel Kir6.2 subunit clone from mouse was generously provided by Dr. S. Seino (Inagaki et al., 1995). The SUR1 subunit clone from hamster was generously provided by Drs. L. Aguilar Bryan and J. Bryan (Aguilar-Bryan et al., 1995). Clones were inserted into the mammalian expression vector pCDNA3. tsA201 cells were plated at 50–70% confluency on 35-mm culture dishes 4 hr before transfection. Clones were then transfected into tsA201 cells using the calcium phosphate precipitation technique as follows. SUR1 (6μg), Kir6.2 (4μg), and pGL (1μg) were diluted in ddH_2O to a final volume of 40μl. 10μl of 2.5M $CaCl_2$ was added along with 50μl 2xHES to yield a final $[Ca^{2+}]$ of 250mM. This reaction mixture was maintained at room temperature for 10 minutes prior to adding drop wise to the plated tsA201 cells. Transfected cells were identified using fluorescence optics in combination with co-expression of the green fluorescent protein plasmid (pGreenLantern, Life Technologies, Inc., Gaithersburg, MD). Single channel recordings were performed 24 h after transfection, macroscopic recordings were performed 48–72 h after transfection. In contrast to cells expressing SUR1/Kir6.2, non-transfected cells did not exhibit any ATP-sensitive potassium current (data not shown).

Molecular Biology. The E23K and I337V mutations were introduced into the Kir6.2 gene by Diana Steckley and Parveen Boora using the protocol outlined in the QuikChange site-directed mutagenesis kit (Stratagene). Briefly, the following primers

were designed from the Kir6.2 cDNA template. For E23K, the forward primer was 5'-AGTACGGTACCTGGGCTTTGCAG-GGTCCTCTGC-3' and the reverse primer was 5'-AAATTTGGTAACACCGTTAAAG-TGCCCACACCA-3'. For I337V, the forward primer was 5'-TGGTGTGGGCACTTTA-ACGGTGTTACCAAATTT-3' and the reverse primer was 5'-GCAGAGGACCCTGCA-AAGCCCAGGTACCGTACT-3'. A 2-step PCR protocol was used to first generate the E23K mutation and then, using the mutated Kir6.2 as template, to generate the double mutant. DpnI digestion was used to remove the wild-type template cDNA. Confirmation of the mutations was achieved through sequence analysis. The ΔC26Kir6.2 truncation mutant was constructed as described previously (Tucker et al., 1997) and mutations introduced using the same protocol.

Electrophysiology. The inside-out patch technique was used to measure macroscopic K_{ATP} channel currents in transfected tsA201 cells. Pipettes were back-filled with solution containing the following (in mM): KCl 134, HEPES 10, $MgCl_2$ 1.4, EGTA 1, KOH 6, glucose 10. The pH of the solution was adjusted to 7.4 with KOH. Macroscopic recordings were performed using patch pipettes pulled from borosilicate glass (G85150T, Warner Instrument Corp., Hamden, CT) to yield pipettes with a resistance of 2 – 6 MΩ. Patch pipettes used for single channel recordings were pulled from borosilicate glass (PG52151-4, World Precision Instruments, Inc., Sarasota, FL) to yield pipette resistances of 20 – 25 MΩ when filled with pipette solution. Once a GΩ seal was formed, the membrane patch was excised from the cell and positioned in the path of a multi-input perfusion pipette. Membrane patches were directly exposed to test solutions under symmetrical K^+ conditions through this perfusion pipette (time to change solution at the tip of the recording pipette was less than 2 sec). All patch clamp experiments were

performed at room temperature (20°–22° C). An Axopatch 200B patch-clamp amplifier and Clampex 8.0 software (Axon Instruments, Foster City, CA) were used for data acquisition and analysis.

Single channel amplitude measurements were analyzed using Fetchan software version 6.0 (Axon Instruments). For measurement of P_o by noise analysis, the following equation was used: $P_o = 1 - [\sigma^2/(i \times I)]$, where σ^2 is the variance measured in the absence of ATP minus the variance measured in between channel opening in the presence of 1mM ATP (i.e. baseline noise when all channels closed), i is the single channel amplitude, and I is the macroscopic mean current measured in the absence of ATP.

Experimental Compounds. MgATP (Sigma, St. Louis, MO) was prepared from a 10 mM stock and stored at –20°C until use. Palmitoyl CoA (Sigma) was dissolved in distilled water as a 5 mM stock solution. Prior to use, stock solutions were sonicated for 5 minutes and diluted in pipette solution to concentrations indicated in text.

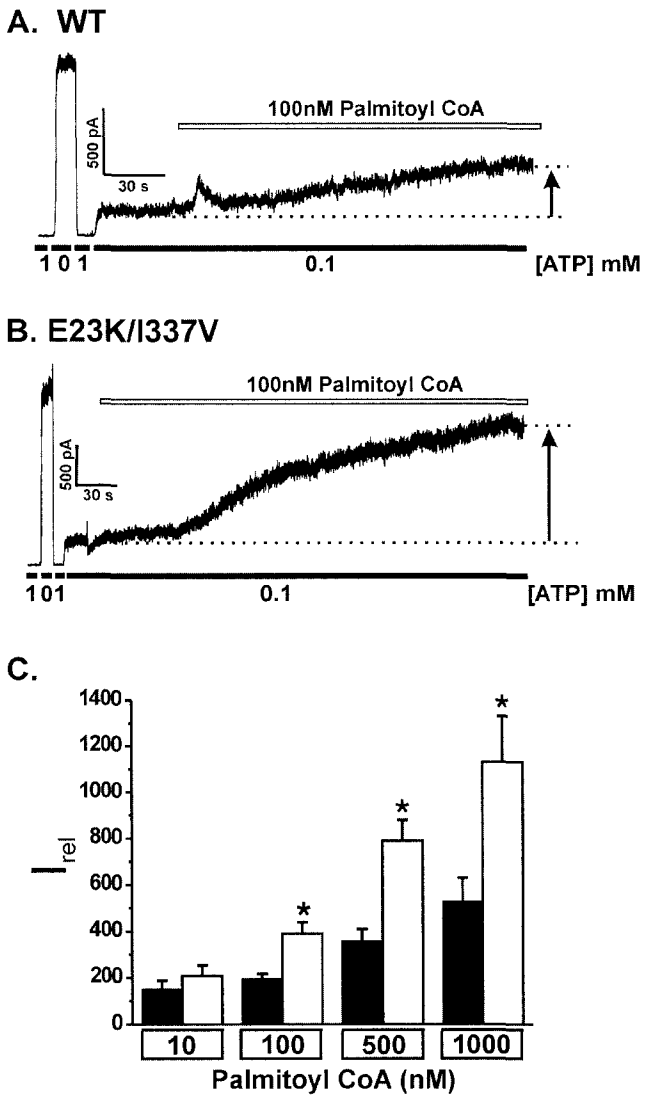
Statistical Analysis. Recombinant macroscopic K_{ATP} currents were normalized to yield I_{rel} where I_{rel} is the current under test conditions relative to the maximal control current observed and expressed as a percentage (i.e. $I_{rel} = I_{test}/I_{control} \times 100$). Statistical significance was assessed using the unpaired Student's t test with P values < 0.05 considered to be significant. Data is expressed as the mean ± SE.

Mathematical Modeling. Simulations of pancreatic β-cell electrical activity were generated by Dr. Gerda de Vries using a modified mathematical model previously described by (Bertram et al., 1995). In this model K_{ATP} channel activity as denoted by conductance is a variable and can thus be modified to reflect the experimentally observed changes in K_{ATP} channel activity. Plateau fraction (Pf) was calculated as follows:

$$Pf = \frac{\text{time spent in plateau phase}}{(\text{time spent in plateau phase} + \text{time spent in silent phase})}$$

and the values then related to equivalent glucose concentrations as described previously (Ozawa and Sand, 1986).

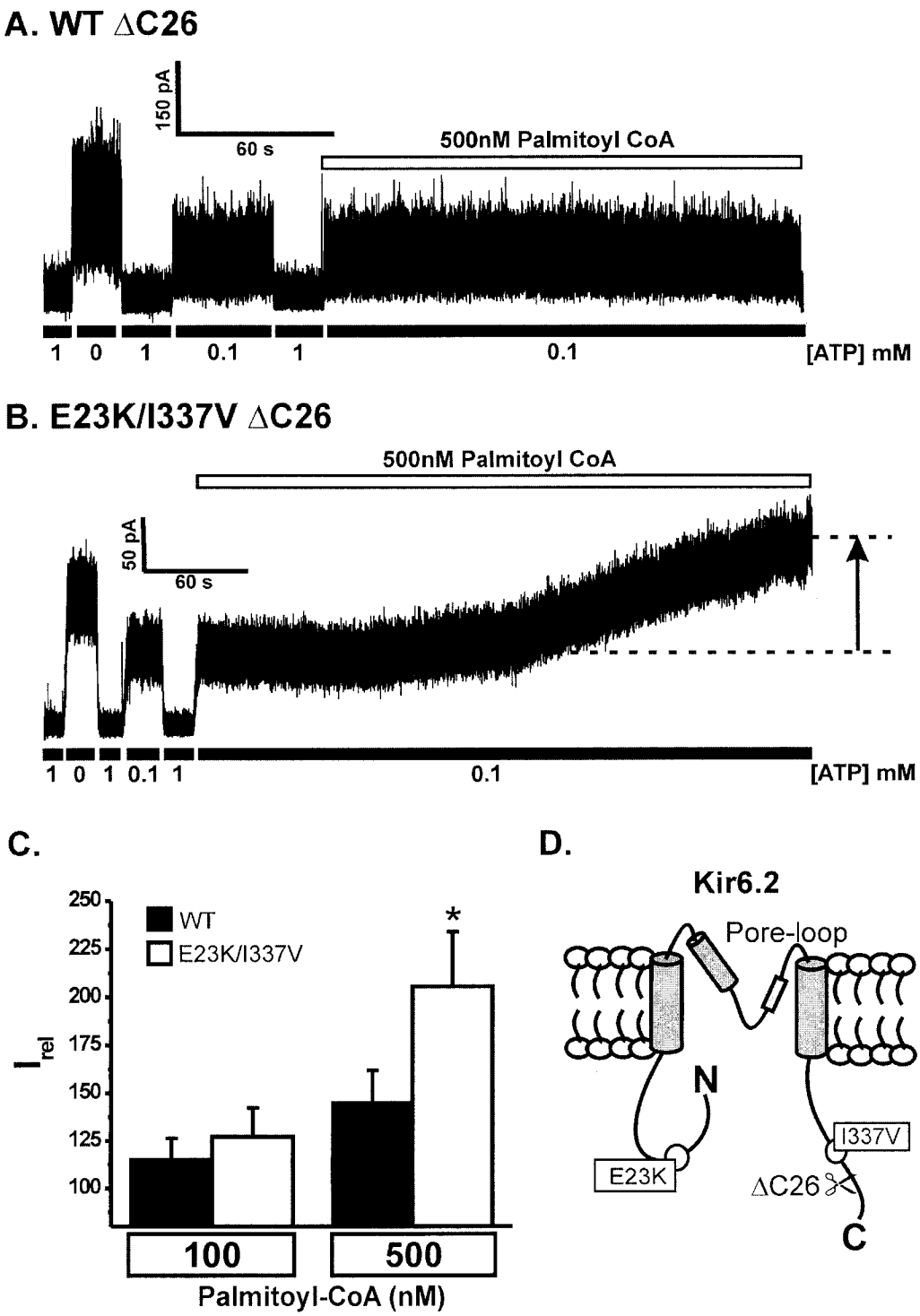
Figure 2-1.



The Kir6.2(E23K/I337V) polymorphism increases K_{ATP} channel sensitivity to long chain acyl CoAs. Representative WT Kir6.2/SUR1 (*A*) and Kir6.2(E23K/I337V)/SUR1 (*B*) channel recordings in response to 100nmol/l palmitoyl-CoA. K_{ATP} currents were recorded at a holding potential of -60 mV under symmetrical K^+ conditions in excised inside-out membrane patches from transiently transfected tsA201 cells (see research design and methods). Dashed lines represent steady-state current levels at 0.1 mM ATP before and after application of palmitoyl-CoA to the inside of the membrane patch. *C.* Histogram plot of pooled data. Values were calculated as steady-state current reached after application of palmitoyl-CoA over steady-state current at 0.1 mM ATP and expressed as mean ± SE. Black bars represent wild-type Kir6.2/SUR1 channel population; white bars represent polymorphic Kir6.2(E23K/I337V)/SUR1 channel population. *$P<0.05$ vs. wild-type pair (n=6-36 patches).

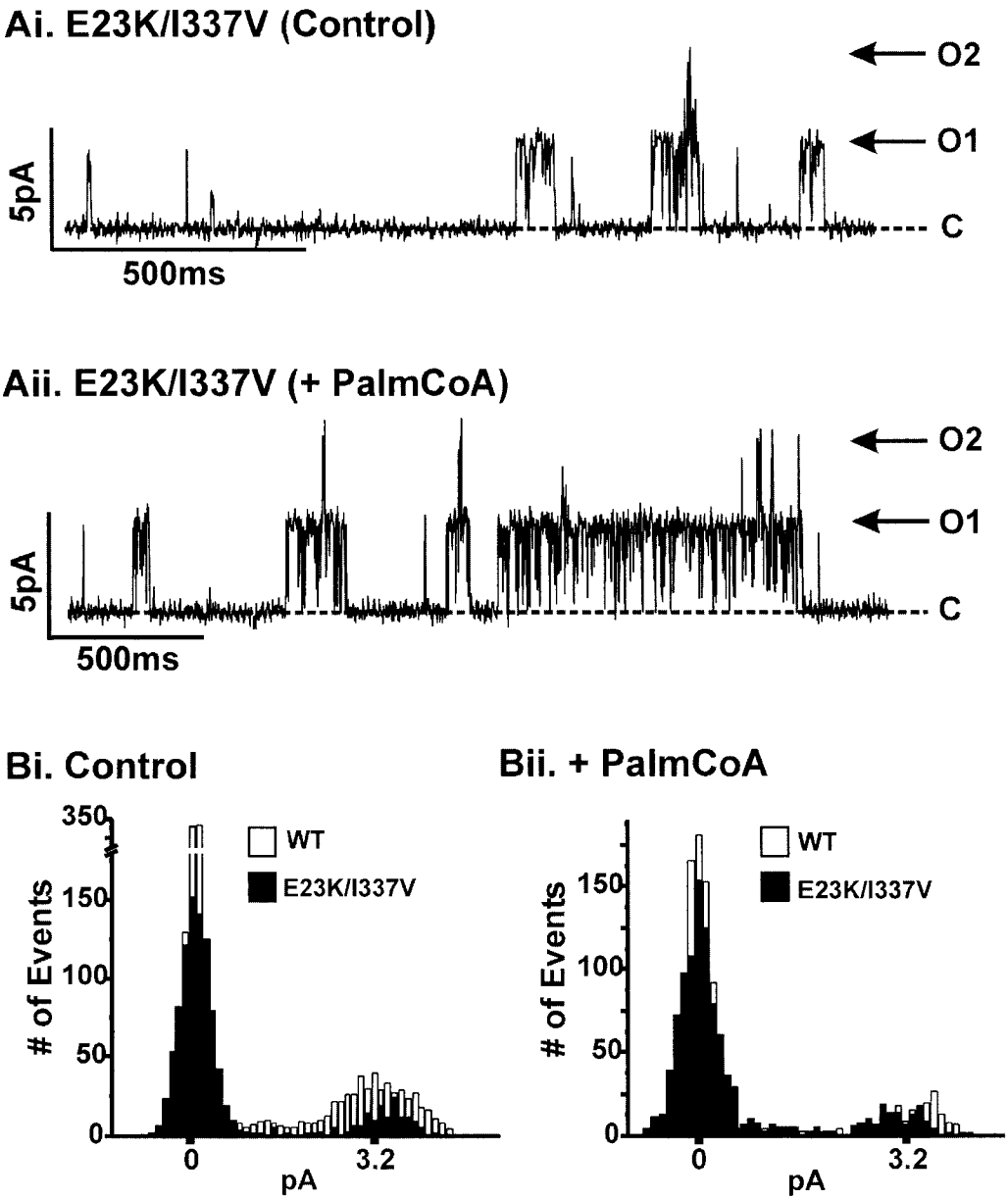
The increase in acyl CoA sensitivity of Kir6.2(E23K/I337V) polymorphic K_{ATP} channels resides primarily in the Kir6.2 subunit. Representative WT Kir6.2ΔC26 (***A***) and polymorphic Kir6.2(E23K/I337V)ΔC26 (***B***) channel recordings in response to 500nM palmitoyl-CoA. K_{ATP} currents were recorded at a holding potential of -60 mV from excised inside-out membrane patches as described previously (see Figure 2-1). Dashed lines represent steady-state current levels at 0.1 mM ATP prior to and after application of palmitoyl-CoA to the inside of the membrane patch. **C:** Histogram plot of pooled data. Values were calculated as steady-state current reached after application of palmitoyl-CoA over the steady-state current at 0.1mmol/l ATP and expressed as mean ± SE. Black bars represent wild-type Kir6.2ΔC26 channel population; white bars represent polymorphic Kir6.2(E23K/I337V)ΔC26 channel population. $*P<0.05$ vs. wild-type pair (n=7-10 patches). ***D.*** Schematic of pore-forming Kir6.2 subunit highlighting the cytosolic location of the E23K and I337V single nucleotide polymorphisms. Removal of the C-terminal 26 amino acids results in the functional expression of Kir6.2 tetrameric K_{ATP} channels.

Figure 2-2.



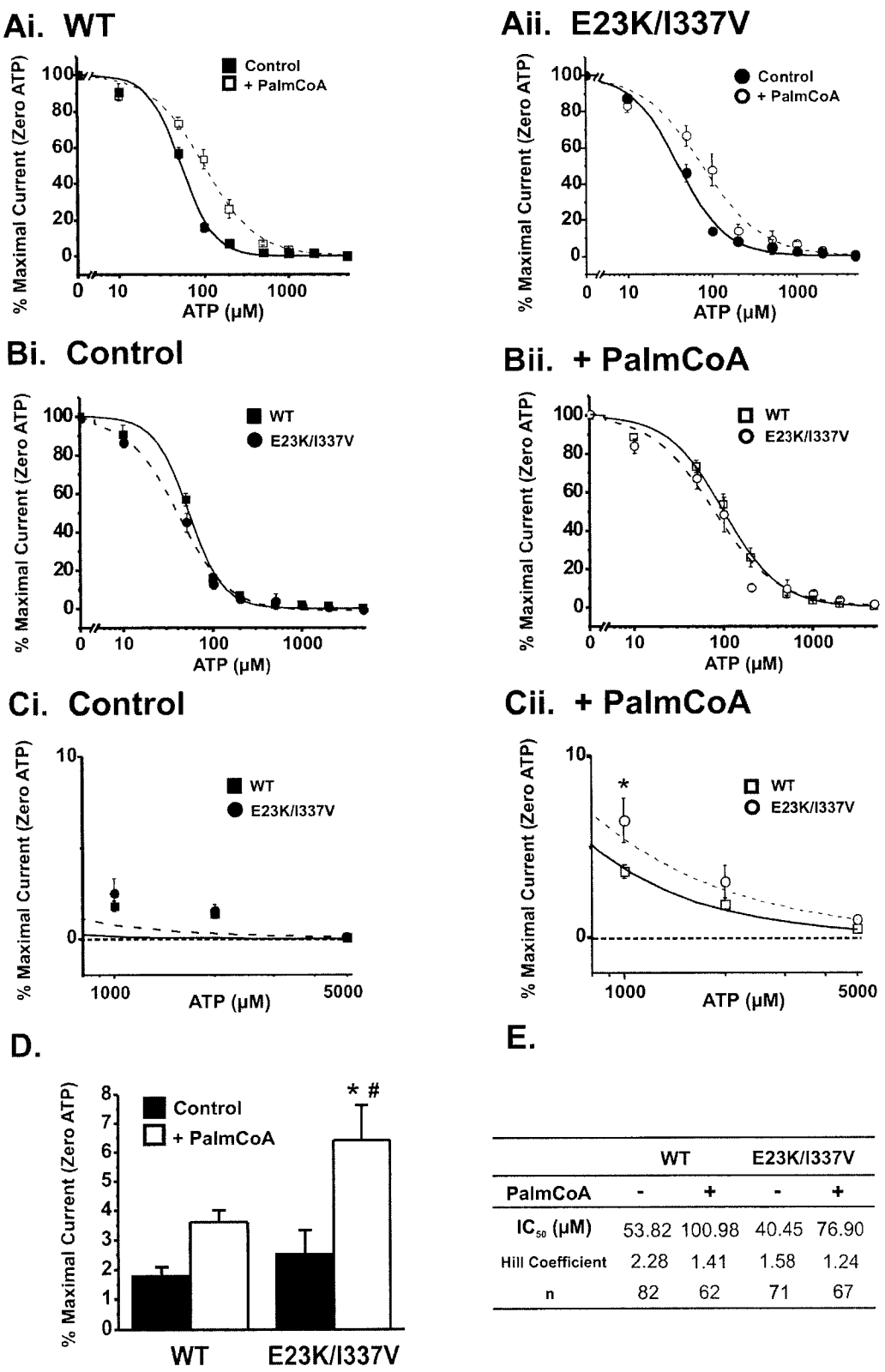
E23K/I337V does not alter single channel amplitude in the absence or presence of Acyl CoAs. ***A.*** Representative single channel current recording from excised inside-out patches of recombinant polymorphic E23K/I337V K_{ATP} channels in the presence of 1 mM ATP prior to (***Ai***) and after application of 100 nM palmitoyl-CoA (***Aii***). C represents the closed channel level; O1 and O2 represent open channel levels with one or two channels open simultaneously, respectively. ***B.*** All points histograms generated from representative single channel recordings indicating that single channel amplitude is unaffected by the E23K/I337V polymorphism (***Bi***), or by the presence of 100 nM palmitoyl-CoA (***Bii***). Black bars represent E23K/I337V polymorphic K_{ATP} channels and white bars represent WT channels. Histograms were plotted on x-axis such that 0 pA level represents peak closed event number.

Figure 2-3.



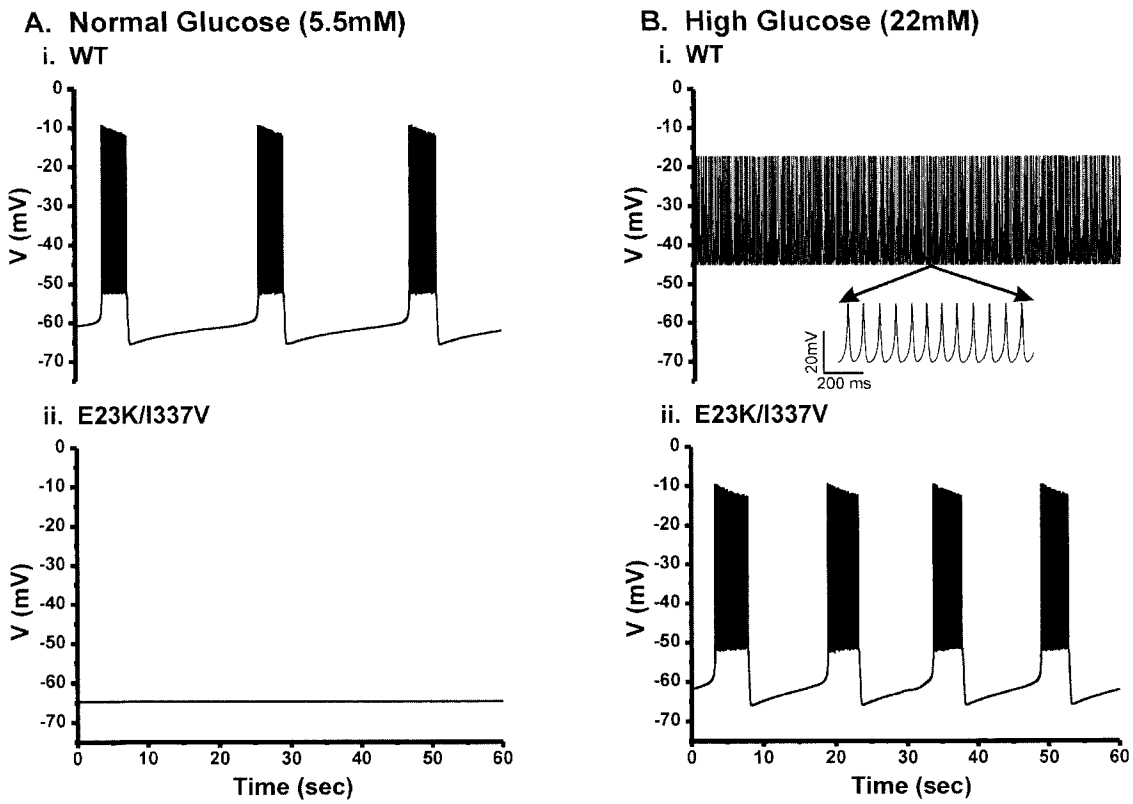
The Kir6.2(E23K/I337V) polymorphism decreases ATP sensitivity of K_{ATP} channels at physiological ATP concentrations. ***A.*** ATP sensitivity of wild-type Kir6.2/SUR1 (**Ai**) and polymorphic Kir6.2(E23K/I337V)/SUR1 (***Aii***) channels in the absence (control) and presence of 100 nM palmitoyl CoA (+ palmCoA). Results are expressed as a percent of maximum current obtained in patches exposed to ATP-free solution. Currents at 5 mM were estimated using Hill function and IC_{50} values reported. ***B.*** ATP Concentration-response curves were plotted as control (***Bi***) and palmitoyl CoA treated (***Bii***) groups to illustrate the effects of the altered Hill coefficient. This reveals the crossover concentrations as detailed in the text. ***C.*** Extrapolated ATP concentration-inhibition curves highlighting physiological range of intracellular ATP. $*P<0.05$ vs. wild-type at 1 mM ATP. ***D.*** Pooled data from ATP concentration-inhibition curves at 1 mM ATP. Black bars represent absence of palmitoyl CoA; white bars represent patches treated with 100nmol/l palmitoyl CoA. Values are expressed as mean ± SE. $*P<0.05$ vs. wild-type patches treated with palmitoyl CoA; $\#P<0.05$ vs. polymorphic channels treated with control solution. ***E.*** Table of results from fitting data in ***Ai*** and ***Aii*** to the equation $I_{rel} = 1/[1 + ([ATP]/IC_{50})^h]$ where I_{rel} is the current observed relative to maximal current in ATP-free solution and h is the Hill coefficient. n values ranged from 8-21 patches for each ATP concentration.

Figure 2-4.



	WT		E23K/I337V	
PalmCoA	-	+	-	+
IC_{50} (μM)	53.82	100.98	40.45	76.90
Hill Coefficient	2.28	1.41	1.58	1.24
n	82	62	71	67

Figure 2-5.



Mathematical simulations of β-cell excitability demonstrate that the observed acyl CoA-induced increases in polymorphic K_{ATP} channel activity at millimolar ATP reduce β-cell bursting behavior. ***A.*** Modeling of pancreatic β-cell membrane potential illustrating bursting electrical behaviour at normal glucose levels (~5.5 mM) incorporating representative conductance values for wild-type (gK_{ATP} = 155pS; ***Ai***) or polymorphic (gK_{ATP} = 185pS; ***Aii***) K_{ATP} channels. ***B.*** Similar illustrations of β-cell electrical activity at elevated glucose levels (~22 mM). K_{ATP} channel conductance values were set at 120pS to represent wild-type K_{ATP} channels (***Bi***) and 150pS to represent polymorphic K_{ATP} channels (***Bii***). Fused bursting of β-cell action potentials is shown in inset of ***Bi*** on an expanded time scale (1 sec). For a detailed description of the mathematical model and parameters used, please refer to Research Design and Methods section.

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Chapter 3

Saturated and *Cis/Trans* Unsaturated Acyl CoA Esters Differentially Regulate Wild-Type and Polymorphic β-Cell ATP-Sensitive Potassium Channels.

Previously published in *Diabetes* under the following citation:

Riedel,M.J., and Light,P.E. (2005). Saturated and *cis/trans* unsaturated acyl CoA esters differentially regulate wild-type and polymorphic β-cell ATP-sensitive potassium channels. Diabetes *54*, 2070-2079.

Introduction

Obesity is a major risk factor for the development of type-2 diabetes. Studies suggest that increasing the amount of dietary energy derived from fat can lead to increased body weight. Coupled with reduced physical activity or energy expenditure, diets high in fat contribute to the development of cardiovascular disease, metabolic syndrome, obesity, and type-2 diabetes (Schrauwen and Westerterp, 2000; Astrup, 2001). Cardiovascular researchers have known for some time that the composition of dietary fat can influence risk for the development of atherosclerosis and coronary artery disease with saturated long chain and *trans* fatty acids having the largest detrimental effect (Ascherio, 2002; Hu et al., 1997; Hu and Willett, 2002). These observations parallel those found in studies linking fat composition to the frequency of type-2 diabetes and suggest a possible link between dietary fat composition and dysfunctional insulin secretion (Salmeron et al., 2001; Meyer et al., 2001). The mechanisms by which dietary fat may alter insulin secretion are not fully characterized, in part due to the dual action of fatty acids on the process of insulin secretion. An acute free fatty acid (FFA) stimulus enhances glucose-stimulated insulin secretion (Stein et al., 1996). However, upon chronic FFA exposure, levels of acyl CoAs, the intracellular esters of FFAs, increase within β-cells contributing to decreased insulin output through several proposed mechanisms (Corkey et al., 2000) including activation of β-cell ATP-sensitive potassium (K_{ATP}) channels.

The appropriate metabolic control of K_{ATP} channels is a critical component of normal GSIS. Under resting conditions, efflux of potassium through K_{ATP} channels maintains the β-cell in a hyperpolarized, inactive state. The metabolic signal initiated by

glucose metabolism is linked to membrane excitability and ultimately the release of insulin through reduction of potassium efflux via closure of K_{ATP} channels.

Despite the large number of K_{ATP} channels (between 1 and 10 thousand per β-cell; Ashcroft and Rorsman, 1989) estimated to populate the surface of an individual β-cell, only ~1% of the total K_{ATP} channel conductance is thought to be available even during resting periods (Cook et al., 1988). As such, only a small number of channels must be closed to initiate insulin secretion. Therefore a relatively small increase in the plasma glucose concentration leading to subtle reductions in K_{ATP} channel activity can evoke a resting β-cell to secrete insulin. Pharmacological agents that inhibit K_{ATP} channels, for example the sulfonylureas, promote insulin secretion whereas activators such as diazoxide limit insulin secretion. Endogenous K_{ATP} channel activators also exist including Mg-ADP, PIP_2, and long chain acyl CoAs. It has previously been shown by our group and others that acyl CoAs can potently activate K_{ATP} channels (see chapter 2 and Riedel et al., 2003; Larsson et al., 1996; Branstrom et al., 1997). Acyl CoAs are amphiphilic molecules comprised of a negatively charged acyl Co-enzyme A head group and a hydrophobic acyl chain. Acyl CoAs are the product of FFA esterification to the CoA moiety by acyl CoA synthetase-1 and thus specific dietary FFAs are directly esterified into acyl CoAs with the same acyl chain structure. Chronic up-regulation of plasma FFA levels, as documented in both obese (Golay et al., 1986) and type-2 diabetic individuals (Reaven et al., 1988), can lead to accumulation of acyl CoAs in the cytosol of pancreatic β-cells (Corkey et al., 2000; Corkey, 1988; Prentki and Corkey, 1996). Because the concentrations of these endogenous K_{ATP} channel activators are elevated in

obesity and type-2 diabetes, it is important to understand the mechanisms by which they contribute to impaired GSIS.

The previously described E23K/I337V K_{ATP} channel polymorphisms have also been shown to alter channel activity and may increase the risk for development of type-2 diabetes in a large subset of the general Caucasian population (for review see Riedel et al., 2005). Evidence suggests that these polymorphisms decrease the ATP sensitivity (Schwanstecher et al., 2002) and increase K_{ATP} channel sensitivity to activation by palmitoyl-CoA (see chapter 2 and Riedel et al., 2003), a common sixteen carbon saturated acyl CoA whose free fat parent molecule is abundant in meat and dairy products.

To date, a comprehensive study on the effects of side chain length, degree of saturation, and *cis/trans* double bond conformation of various acyl CoAs has not been performed. Therefore, it was the aim of this study to investigate the K_{ATP} channel-activating properties of various common dietary acyl CoAs and to establish a molecular model to explain the process of acyl CoA-mediated channel activation. Our results indicate that longer chain saturated and *trans* fatty acids exert a larger stimulatory effect on the K_{ATP} channel, and that the ability of any given acyl CoA to activate the channel is directly related to its hydrophobicity.

Results

Long chain acyl CoAs activate β-cell K_{ATP} channels in a side-chain length and degree of saturation-dependent manner.

K_{ATP} channel activity was measured from excised membrane patches containing the recombinant β-cell isoform Kir6.2/SUR1 (Figure 3-1). Wild-type (Figure 3-1A) K_{ATP}

channels were activated by physiological free concentrations of palmitoyl CoA, stearoyl CoA, and oleoyl CoA (Table 3-1). In accordance with our previously published results (Riedel et al., 2003 and see chapter 2), application of these long chain acyl CoAs to E23K/I337V polymorphic K_{ATP} channels resulted in a significantly larger increase in current versus wild-type (Figure 3-1B). The increase in chain length from 16 carbons to 18 carbons (palmitoyl CoA vs. stearoyl CoA) resulted in a significant increase in channel activity (Figure 3-1C). Conversely, introduction of one (palmitoleoyl CoA and oleoyl CoA) or two (linoleoyl CoA) double bonds reduced this stimulatory effect. Acyl CoAs that did not evoke a high level of wild type K_{ATP} channel activation (palmitoleoyl CoA and linoleoyl CoA) similarly did not result in a significantly greater activation in polymorphic K_{ATP} channels.

Trans-acyl CoAs and activation of the K_{ATP} channel.

To assess the importance of the *cis* vs. *trans* double-bond conformation, we activated K_{ATP} channels with both elaidoyl CoA (*trans*-Δ^9-octadecanoyl CoA) and the *cis* conformation of the same acyl CoA, oleoyl CoA (*cis*-Δ^9-octadecanoyl CoA). Elaidoyl CoA and oleoyl CoA activated the K_{ATP} channel to a similar extent at a concentration of 100 nM. Again, E23K/I337V polymorphic K_{ATP} channels were activated significantly more compared to wild-type K_{ATP} channels (Figure 3-2C).

The overall stimulatory effect of acyl CoAs on the K_{ATP} channel is likely a function of both their ability to maximally activate the channel (efficacy) and their ability to access the binding site (affinity). Efficacy was assessed by the maximum activation effect as plotted in figure 3-1, while affinity was determined in two steps (Figure 3-3). First, we measured the time required for the acyl CoA stimulus to achieve 75% of the

steady-state K_{ATP} channel activation (Figure 3-3C). Our results show that elaidoyl CoA took slightly, but not significantly longer to act than oleoyl CoA (P=0.08) suggesting that bond conformation may play a small role in determining binding affinity. Similarly, stearoyl CoA required more time than oleoyl CoA or linoleoyl CoA to achieve its maximum efficacy. Secondly, we measured the ability of acyl CoAs to unbind from the channel by perfusing the membrane patch with an acyl CoA-free control solution following the initial acyl CoA stimulus (Figure 3-3). Application of oleoyl CoA to the membrane patch resulted in significant K_{ATP} channel activation as detailed above (Figure 3-1). Upon removal of oleoyl CoA, the membrane current decreased rapidly and approached control levels within 5 minutes (Figure 3-3A). Conversely, exposure of the membrane patch to elaidoyl CoA resulted in a sustained, persistent K_{ATP} channel activation over the course of the experiment (Figure 3-3B). Similar persistent K_{ATP} channel activation was observed with both palmitoyl CoA and stearoyl CoA, the two fully saturated acyl CoAs used in this study (Figure 3-3D).

Hydrophobic or amphiphilic molecules such as FFAs and acyl CoAs respectively, can partition into biological membranes, potentially altering physical characteristics such as membrane fluidity and integrity (Cohen et al., 2003). Increases in membrane fluidity can cause increased leak current which could be misinterpreted as an increase in K_{ATP} channel activity. Therefore, the above observation was confirmed at a lower concentration of 100 nM to insure that the persistent K_{ATP} channel activity was not a result of an increase in leak current caused by disruption of membrane integrity at higher acyl CoA concentrations.

Acyl CoAs of differing side chain composition may compete for a shared binding site on the K_{ATP} channel.

A subsequent series of experiments was performed to test the effects of simultaneous application of two acyl CoAs with different K_{ATP} channel stimulatory properties. Stearoyl CoA and linoleoyl CoA were used due to the large difference in levels of channel activation observed at 100 nM (8.7 ± 0.7 vs. 2.9 ± 0.3 fold respectively). While maintaining a constant total acyl CoA concentration at 100 nM, increasing the relative ratio of linoleoyl CoA to stearoyl CoA from 1:1 to 2:1 resulted in a reduction of maximum normalized current to 5.3 ± 0.2 fold (Figure 3-4A). A further increase in the linoleoyl CoA to stearoyl CoA ratio to 5:1 resulted in an additional decrease in K_{ATP} channel activation to 3.2 ± 0.6 fold, a level of activation not significantly different to that of linoleoyl CoA alone.

To confirm that the above observation did not simply represent a concentration dependence of K_{ATP} channel activation by stearoyl CoA, membrane patches were pre-exposed to 500 nM linoleoyl CoA prior to challenging the channels with a 1:5 ratio of stearoyl CoA to linoleoyl CoA (500 nM total acyl CoA concentration). Linoleoyl CoA alone resulted in an increase in K_{ATP} channel activity of 2.3 ± 0.4 fold. The subsequent addition of 100 nM stearoyl CoA in the continued presence of linoleoyl CoA resulted in a much higher level of K_{ATP} channel activity (9.5 ± 3.5 fold) however two distinct observations were made: *1)* the ability of stearoyl CoA to activate K_{ATP} channels was delayed. Figure 3-4Bi shows the time required from the application of stearoyl CoA to reach 25% of the maximum attained current. This time was significantly increased in membrane patches that had been pre-exposed to 500 nM linoleoyl CoA relative to those

that had been exposed to stearoyl CoA alone (105.59 ± 8.00 sec vs. 59.46 ± 14.85 sec). *2)* the maximum slope of activation (V_{max}) was significantly reduced in these membrane patches (Figure 3-4Bii). Taken together, these results suggest that prior to activating the K_{ATP} channel, stearoyl CoA must first displace linoleoyl CoA, perhaps due to interactions with a shared binding site(s) on the K_{ATP} channel itself.

In another experiment, the membrane patch was pre-exposed to 10 nM stearoyl CoA to elicit significant K_{ATP} channel activation. The effects of either 100 or 500 nM linoleoyl CoA were then tested in the continued presence of stearoyl CoA. Under these conditions linoleoyl CoA failed to reduce the stimulatory effect of stearoyl CoA (Figure 3-4C). This suggests that, once bound, stearoyl CoA is unable to be competitively displaced, even by saturating concentrations of linoleoyl CoA.

The hydrophobicity of the acyl chain predicts the efficacy of acyl CoAs on K_{ATP} channel activation.

We plotted the normalized K_{ATP} channel current in the presence of each tested acyl CoA against its partition coefficient between octanol and water (Figure 3-5A). Partition coefficients (Log P values) were obtained online from the Syracuse Research Corporation Physical Properties Database (PHYSPROP) for FFAs with carbon chain lengths ranging from 8 to 20. A single exponential was fitted to the data obtained from this study and extrapolated to include log P values below that of palmitoleoyl CoA. Interestingly, as acyl chain length or degree of saturation increases, the log P value also increases, with stearoyl CoA having the longest and most saturated acyl tail as well as the largest log P value. Also plotted in figure 3-5 are results obtained from two previous studies (Fox et al., 2003; Branstrom et al., 2004). Data from Branstrom and colleagues

includes octanoyl CoA (C8:0), lauroyl CoA (C12:0), and myristoyl CoA (C14:0) while data from Fox and colleagues adds decanoyl CoA (C10:0) to the data-set. It has been previously shown that acyl CoAs with chain lengths below 14 do not significantly activate K_{ATP} channels (Larsson et al., 1996). This may result from their low partition coefficient and therefore a reduced ability to incorporate into hydrophobic environments such as the lipid bilayer.

Figure 3-5B shows the correlation between acyl chain length and K_{ATP} channel activation. Both saturated (Figure 3-5Bi) and monounsaturated (Figure 3-5Bii) acyl CoAs activate the K_{ATP} channel in a manner highly correlated to chain length. These results show that even small changes in the Angstrom (Å) length of the acyl chain such as exists between oleoyl CoA and elaidoyl CoA can alter the effect on K_{ATP} channel activity, despite these acyl CoAs having similar Log P values. This suggests that the physical properties of the acyl chain including, but not limited to its length, are important in determining the overall efficacy of any given acyl CoA on K_{ATP} channel activity.

Taking the above observations into consideration, we propose a model whereby the negatively charged CoA head group binds to a specific site(s) on the K_{ATP} channel while the acyl tail partitions into the plasma membrane. Our observations indicate that saturated and long chain acyl CoAs cause a large but slowed stimulatory response and do not readily unbind from the channel (Figure 3-6A). In contrast, shorter chain and unsaturated acyl CoAs, which have lower partition coefficients and are more mobile in hydrophobic environments, activate K_{ATP} channels to a lesser extent, an effect that is readily reversible (Figure 3-6B).

Discussion

The results from this study demonstrate that acyl CoAs modulate K_{ATP} channel activity in a manner dependent on the hydrophobic acyl chain length as well as the degree of saturation. We also provide evidence consistent with the notion that these lipid modulators may interact with a specific binding site constituting the channel protein itself as well as the lipid bilayer. In addition, we confirm previous findings indicating that the common E23K/I337V polymorphism increases the susceptibility of the K_{ATP} channel to activation by acyl CoAs (Riedel et al., 2003 and see chapter 2).

Mechanisms of acyl CoA-mediated K_{ATP} channel activation

Lipid modulation is thought to play a major role in fine-tuning K_{ATP} channel activity *in vivo*, given that under physiological nucleotide levels more than 99% of plasma membrane K_{ATP} channels are thought to be closed (Cook et al., 1988). Acyl CoAs may represent one of the most important classes of lipid modulators with a similar efficacy but greater potency and selectivity for K_{ATP} channels than the anionic phospholipid PIP_2 (Liu et al., 2001; Rohacs et al., 2003). In pathological situations such as obesity (Golay et al., 1986) and type-2 diabetes (Reaven et al., 1988), high circulating levels of FFAs lead to cytosolic accumulation of acyl CoAs (Corkey et al., 2000) indicating that this class of ion channel modulators may play a contributory role in the mechanism by which obesity precipitates dysfunctional GSIS and increases susceptibility to type-2 diabetes.

In the current study, we have examined the effects of various acyl CoAs on K_{ATP} channel activity using physiological free concentrations approximately 100 times below the critical micellar concentration (Cohen et al., 2003). While all acyl CoAs tested in this

study increased K_{ATP} channel activity, our results indicate that the general rank order of efficacy was saturated > monounsaturated > n-6 polyunsaturated acyl CoAs. We also found that longer chain acyl CoAs activated the K_{ATP} channel to a greater extent than shorter chain acyl CoAs. These data are consistent with other reports indicating that increased acyl CoA side-chain length and saturation leads to increased K_{ATP} channel activity (Larsson et al., 1996; Branstrom et al., 2004; Branstrom et al., 1998). However, based on results indicating that palmitoyl CoA and oleoyl CoA elicited similar K_{ATP} channel activation, previous studies have suggested that this stimulatory effect is maximal at 16 carbons in side-chain length (Branstrom et al., 2004). Our data is in agreement with this observation as these two acyl CoAs have a similar hydrophobicity (Figure 3-5A). However, the fully saturated 18 carbon stearoyl CoA clearly elicited a greater response in both wild-type and E23K/I337V polymorphic K_{ATP} channels compared to palmitoyl CoA and oleoyl CoA.

Interestingly, the ability of a particular acyl CoA to activate the K_{ATP} channel may be directly related to its ability to partition into hydrophobic environments. This is the case not only with the acyl CoAs used in this study but also for acyl CoAs of shorter chain length used in previous studies (Fox et al., 2003; Branstrom et al., 2004). Using the partition coefficient between water and octanol as an indication of hydrophobicity, we fit a single exponential curve to our K_{ATP} channel activation data that allows us to predict the effect of a given acyl CoA on the channel based on acyl chain hydrophobicity. At this point it is unclear as to whether or not the relationship holds true for acyl CoAs of chain length greater than 20. Further studies examining both natural and synthetic very long chain acyl CoAs will be required to probe the upper limits of our prediction.

An examination of the effect of *trans* fatty acids on the activity of K_{ATP} channels yielded interesting results. Although elaidoyl CoA and oleoyl CoA activated the K_{ATP} channel to a similar extent, our data shows that K_{ATP} channels exposed to the *trans* 18 carbon monounsaturated elaidoyl CoA remained active for an extended period of time relative to the *cis* isoform oleoyl CoA once the acyl CoA stimulus was removed. This important observation reveals a possible mechanism by which *trans* monounsaturated fatty acids may be more detrimental to insulin secretion than the corresponding *cis* monounsaturated fatty acid. Prolonged channel activation could have a similar effect to that of an increased maximum response such as occurs in the presence of saturated acyl CoAs in maintaining a hyperpolarized β-cell membrane and reduced glucose responsiveness.

The specific interaction of long chain acyl CoAs with the K_{ATP} channel is not currently well understood. A binding region for the CoA head group has been suggested to reside in the Kir6.2 subunit (Schulze et al., 2003; Manning Fox et al., 2004) but a precise mechanism detailing how binding of this head group and the associated acyl chain leads to stabilization of the open state is lacking. We have attempted to further our understanding of the nature of this interaction by investigating the importance of acyl CoA chain length and saturation. Our data shows that acyl CoA efficacy and affinity is affected by the acyl chain structure (Figure 3-3C,D). In addition, we found that *1)* binding of polyunsaturated acyl CoAs could delay the activation efficacy of saturated acyl CoAs (Figure 3-4A) and *2)* persistent K_{ATP} channel activation induced by stearoyl CoA could not be diminished by a saturating concentration of the weakly activating linoleoyl CoA (Figure 3-3C). Taken together, these data suggest that a shared binding

site may be involved in the activation by various acyl CoAs and that this may be due to binding of the common CoA head group shared amongst each acyl CoA (see below). If the shared binding site(s) is initially unoccupied then saturated and unsaturated acyl CoAs are equally able to occupy the vacant site(s) as they share the identical CoA moiety (Figure 3-4A). However, once bound, saturated acyl CoAs are unable to be competed off by unsaturated acyl CoAs, providing further evidence for the involvement of the acyl chain in the persistence and magnitude of activation. The slower response and persistent activation observed with saturated and *trans* monounsaturated acyl CoAs (Figure 3-3C,D) may reflect a reduced lateral mobility of the saturated acyl chain within the membrane.

Additional support for this model comes from studies of acyl CoA incorporation into artificial membrane preparations. It has been previously shown that these amphiphilic molecules associate with membranes through insertion of the fatty acyl chain (Cohen et al., 2003) and that this interaction becomes stronger with increased side-chain length (Requero et al., 1995). Knudsen's group has also shown that lateral diffusion of acyl CoAs occurs in membranes leading to the formation of aggregates near areas of increased membrane curvature (Cohen et al., 2003) which may include membrane spanning proteins such as ion channels. Therefore it is plausible that local acyl CoA concentrations may be higher in the proximity of K_{ATP} channels than in the surrounding membrane. In addition, incorporation of different acyl CoAs into the membrane may be constant but the reduced lateral diffusion rate of saturated and *trans* acyl CoAs may increase the longevity of channel opening by maintaining the CoA head group in close proximity to its binding site on the K_{ATP} channel as evidenced in figure 3-3B,D. Reduced

lateral diffusion may also result in the increased time to activation and reduced washout of saturated and *trans* acyl CoAs as observed in this study.

Given the similarity in proposed crystal structure of the trans-membrane domain of KcsA and KirBac1.1 and their sequence and structural homology with Kir6.2, speculation can be made on potential interactions of acyl CoAs with Kir6.2 based on previous work performed on these related potassium channels (Williamson et al., 2003). We have previously identified several positively-charged residues in both the C-terminal and N-terminal domains of the cytosolic portion of the Kir6.2 subunit that are important for acyl CoA binding. In particular, residue R54 from one Kir6.2 subunit is thought to interact with R176 and R301 of the adjacent subunit to form a positively-charged region to which the negatively charged CoA head group may bind (Manning Fox et al., 2004). The exact placement of the head group in this region is currently unknown but it appears that binding may position the fatty acyl chain to interact with the plasma membrane. Previous work by Williamson et al. (Williamson et al., 2003) on the KcsA bacterial potassium channel indicates that fatty acids can bind to the α-helices that contain the channel gate. Depending on the nature of this interaction, binding of fatty acids to these helices will likely result in alterations in their spatial arrangement and may lead to opening or closing of this gate. This fatty acid/α-helix interaction is highly dependent on the chain length. Binding occurs with a minimum acyl chain length of 10 carbons and becomes optimal at a tail length of 22 carbons (Williamson et al., 2002). This matches very closely with our data showing an effect on K_{ATP} channel activity with acyl chains of 14 carbons or greater (Figure 3-5; Larsson et al., 1996; Branstrom et al., 1998) and is in agreement with our data indicating that the activation of K_{ATP} channels by acyl CoAs

does not saturate with the 16 carbon palmitoyl CoA but is greater with the 18 carbon stearoyl CoA. Further studies to probe the binding of acyl chains to α-helices within the K_{ATP} channel are warranted to assess the similarity between fatty acid binding to KcsA and Kir6.2. The fact that the 10 carbon decanoyl CoA does not activate the β-cell K_{ATP} channel may be as a result of the CoA binding site (including residues R54 and R176) lying below the plane of the membrane, necessitating a longer acyl chain to significantly partition into the lipid bilayer, or through additional complexities of binding due to interactions with the SUR1 subunit. More detailed molecular modeling of CoA binding including proper placement of the head group within this region will be required before a firm mechanism for acyl CoA-mediated K_{ATP} channel activation can be established.

Clinical Relevance

In both human and animal studies, high fat diets routinely lead to insulin resistance with most leading to dysfunctional GSIS (Wilkes et al., 1998; Dobbins et al., 2002; Zhou and Grill, 1995; Zhou and Grill, 1994). The observed inconsistency with regards to insulin secretion may result from two mechanisms. First, there is a biphasic effect of fatty acids on insulin secretion with an early stimulatory effect followed by a secondary inhibitory action that occurs with chronic fat exposure (Carpentier et al., 1999). Secondly, most studies have examined the effects of a mixture of fatty acids on insulin secretion with the percentages of each fat type (saturated vs. mono- or polyunsaturated) modified to reflect the nature of the study (Dobbins et al., 2002). However, two recent large scale prospective studies support our hypothesis that saturated and *trans* fatty acids are more detrimental to GSIS than unsaturated fatty acids. The Iowa Women's Health Study of ~36,000 women found that the incidence of type-2 diabetes

was positively associated with higher intake of saturated animal fats and negatively associated with higher intake of unsaturated vegetable fats (Meyer et al., 2001). These results are similar to those found in the Finnish and Dutch cohorts of the smaller Seven Countries Study (Feskens et al., 1995). In addition, the Nurses' Health Study examined ~84,000 women, concluding that the incidence of type-2 diabetes was greater in those ingesting higher amounts of *trans* fatty acids and lower in those with a moderate increase in polyunsaturated fatty acid intake (Salmeron et al., 2001).

Summary

In conclusion, we have shown that the direct exposure of K_{ATP} channels to long chain acyl CoAs leads to increased channel activity in a manner dependent on both acyl chain length and degree of saturation. Saturated acyl CoAs are the most effective activators of K_{ATP} channels followed by monounsaturated and polyunsaturated acyl CoAs respectively. The *trans* monounsaturated elaidoyl CoA activated K_{ATP} channels with similar efficacy to its *cis* monounsaturated counterpart (oleoyl CoA) but did not unbind from the channel as readily. This may result in a prolonged activation of K_{ATP} channels exposed to this and perhaps other as yet untested *trans* acyl CoAs, contributing to reduced β-cell excitability and subsequent reductions in insulin secretion. Our results also indicate that different acyl CoAs can compete for a binding region on the K_{ATP} channel and that an advantage is given to those acyl CoAs with higher partitioning coefficients or reduced ability to unbind from or laterally move within the membrane. Finally, we have expanded on our previous findings (Riedel et al., 2003 and see chapter 2) showing that E23K/I337V polymorphic K_{ATP} channels are more sensitive to activation by long chain saturated and monounsaturated acyl CoAs compared to wild-type channels.

Our data lends support to previous clinical studies that suggest replacement of saturated fat with either monounsaturated or polyunsaturated fat as a means to improving glucose tolerance in healthy (Uusitupa et al., 1994) and glucose-intolerant subjects (Vessby et al., 1980) and reducing the incidence of newly diagnosed type-2 diabetes (Salmeron et al., 2001) and has implications for dietary management of obese or type-2 diabetic individuals who are homozygous polymorphic for the E23K polymorphism.

Research Design and Methods

Molecular Biology. The K_{ATP} channel Kir6.2 subunit clone from mouse, kindly provided by Dr. S. Seino and the SUR1 clone from hamster, kindly provided by Drs. L. Aguilar Bryan and J. Bryan were used in this study. Clones were inserted into the mammalian expression vector pcDNA3. The E23K and I337V mutations were introduced into the Kir6.2 gene using the protocol outlined in the QuikChange site-directed mutagenesis kit (Stratagene) as previously described (see chapter 2). The generation of mutations was confirmed through sequence analysis.

Cell Culture and Transfection. tsA201 cells (an SV40-transformed variant of the HEK293 human embryonic kidney cell line) were maintained in Dulbeco's Modified Eagle's medium supplemented with 25 mM glucose, 2 mM L-glutamine, 10% FCS, and 0.1% penicillin/streptomycin in a humidified incubator at 37°C with 5%CO_2. Cells were passaged and plated at 50 – 70% confluency on 35 mm culture dishes approximately 4 hours prior to transfection. Clones were then transfected into the tsA201 cells using the calcium phosphate precipitation technique as detailed in chapter 2. Transfected cells were identified using fluorescent optics in combination with co-expression of the green fluorescent protein plasmid (pGreenLantern; Life Technologies, Gaithersburg, MD).

Macroscopic K_{ATP} channel recordings were then performed 48 – 72 hours after transfection.

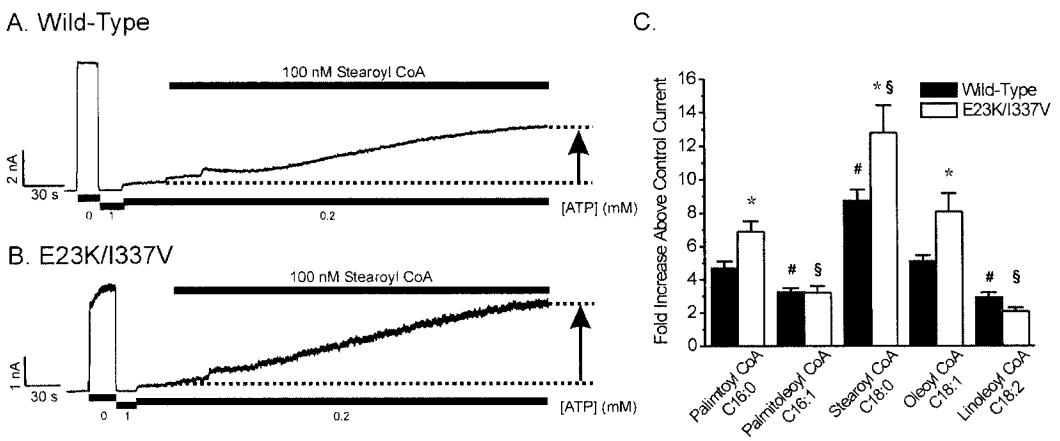
Electrophysiology. The inside-out patch clamp technique was used to measure macroscopic K_{ATP} channel currents in transfected tsA201 cells. Patch pipettes were pulled from borosilicate glass (G85150T; Warner Instruments, Hamden, CT) to yield resistances between 2 and 6 MΩ when back-filled with a buffer solution containing the following (in mM): 110 KCl, 30 KOH, 10 EGTA, 5 HEPES, and 1 $MgCl_2$. The pH of the solution was adjusted to 7.4 with KOH. Once a GΩ seal was formed, the membrane patch was excised from the cell and positioned in the path of a multi-input perfusion pipette. The cytosolic face of the membrane patch was held at a holding potential of -60 mV to elicit inward K_{ATP} channel currents and the resulting upward current deflections were plotted using Origin graphing software (Microcal Software, Inc., Northampton, MA). Membrane patches were directly exposed to test solutions under symmetrical K^+ conditions through this perfusion pipette (time to change solution at the tip of the recording pipette was <2 sec.). All patch clamp experiments were performed at room temperature (20 – 22°C). An Axopatch 200B patch clamp amplifier and Clampex 8.0 software (Axon Instruments, Foster City, CA) were used for data acquisition and analysis.

Experimental Compounds. Mg-ATP (Sigma, Oakville, ON) was prepared as a 10 mM stock and stored at -20°C until use. Long chain acyl CoAs palmitoyl CoA (C16:0), *cis*-Δ^9-palmitoleoyl CoA (C16:1), stearoyl CoA (C18:0), *cis*-Δ^9-oleoyl CoA (C18:1cis), *trans*-Δ^9-elaidoyl CoA (C18:1trans), *cis,cis*-$\Delta^{9,12}$-linoleoyl CoA (C18:2), and *all cis*-$\Delta^{5,8,11,14}$-arachidonyl CoA were purchased from Sigma as Li^+ salts and dissolved in

ddH_2O as 1 mM stock solutions. Prior to use, stock solutions were sonicated for 5 min and diluted in pipette solution to concentrations indicated in the text.

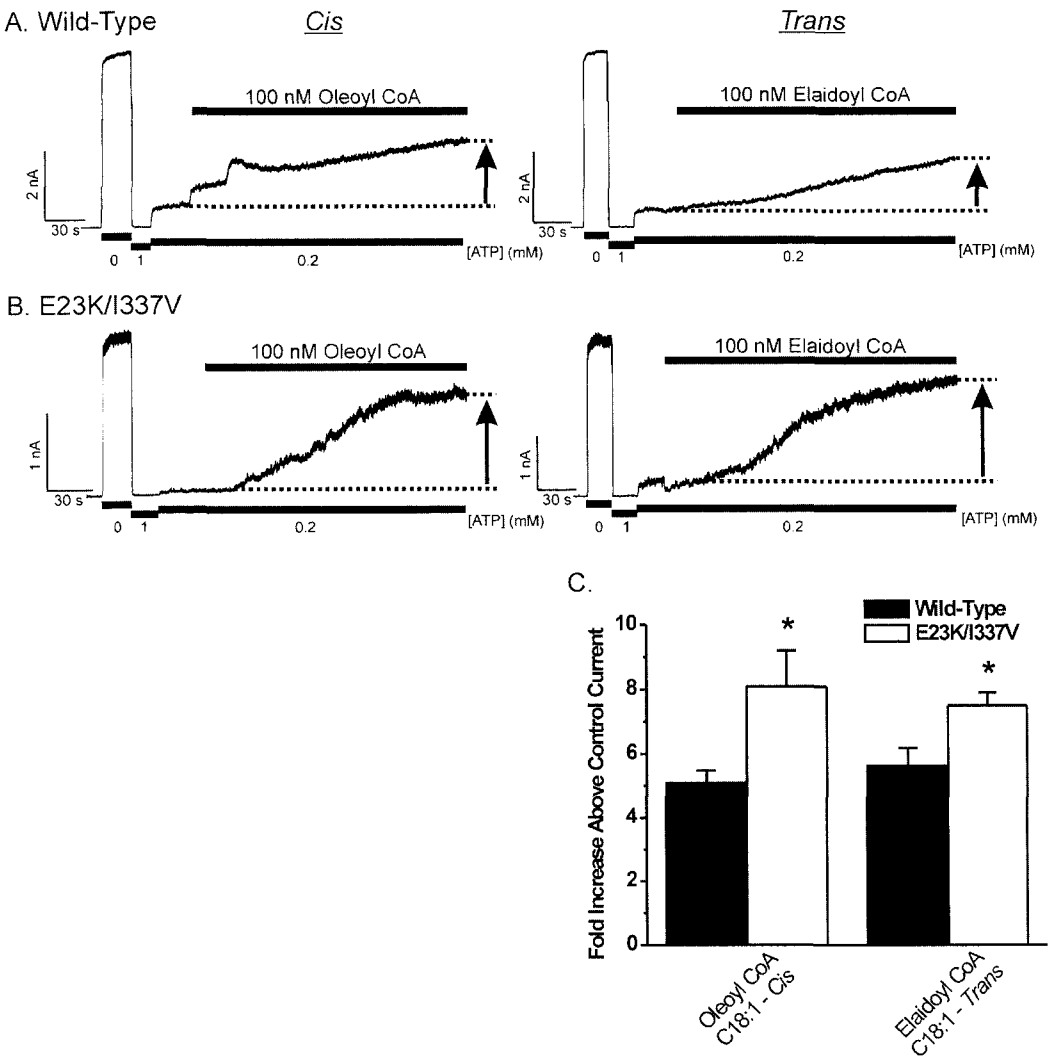
Statistical Analysis. Recombinant macroscopic K_{ATP} channel currents were normalized and expressed as an increase in current relative to control (i.e., normalized K_{ATP} channel current = $I_{test}/I_{control}$) where I_{test} is the current elicited by the acyl CoA stimulus and $I_{control}$ is the current elicited by 0.2 mM MgATP. Statistical significance was assessed using the unpaired Student's t test, with $P < 0.05$ considered statistically significant. Data are expressed as the mean ± SE.

Figure 3-1.



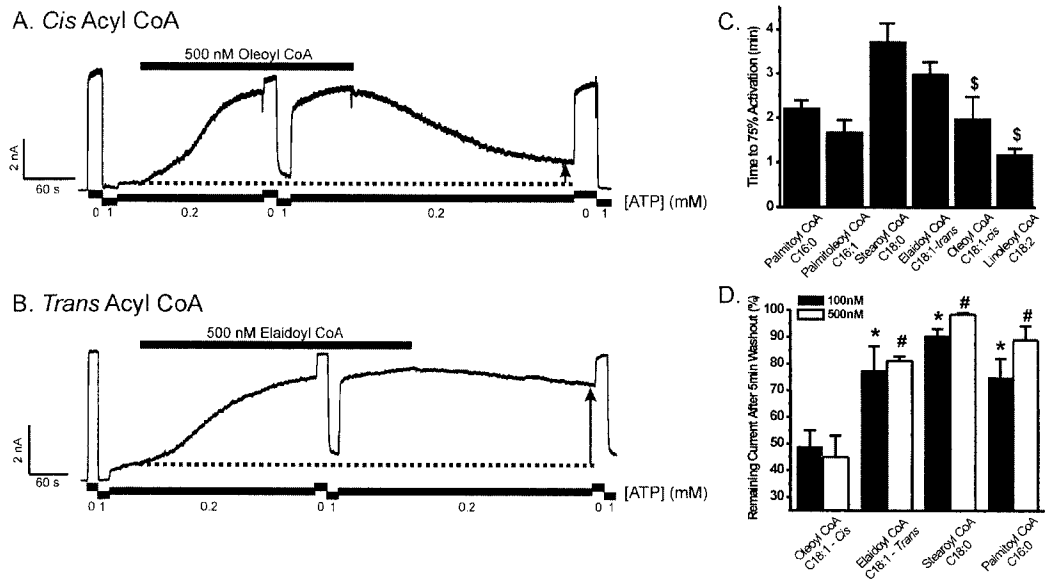
Activation of wild-type and E23K/I337V polymorphic K_{ATP} channels by acyl CoAs is dependent on both acyl CoA chain length and degree of saturation. ***A*** and ***B.*** Representative wild-type Kir6.2/SUR1 (***A***) and Kir6.2(E23K/I337V)/SUR1 polymorphic (***B***) K_{ATP} channel recordings in response to 100 nM stearoyl CoA. K_{ATP} channel currents were recorded at a holding potential of -60 mV under symmetrical K^+ conditions in excised inside-out membrane patches from transiently transfected tsA201 cells (see Research Design and Methods). Dashed lines represent steady-state current levels at 0.2 mM Mg-ATP prior to and following application of stearoyl CoA to the inside of the membrane patch. ***C.*** Compiled data on acyl CoA chain length and degree of saturation. Values were calculated as steady-state current reached following application of the given acyl CoA over the steady-state current at 0.2 mM MgATP and expressed as mean ± SE. $*P < 0.05$ versus corresponding wild-type pair; $\#P < 0.05$ vs. palmitoyl CoA in wild-type K_{ATP} channel populations; $\S P < 0.05$ vs. palmitoyl CoA in E23K/I337V polymorphic K_{ATP} channel populations (n = 5-19 patches per group).

Figure 3-2.



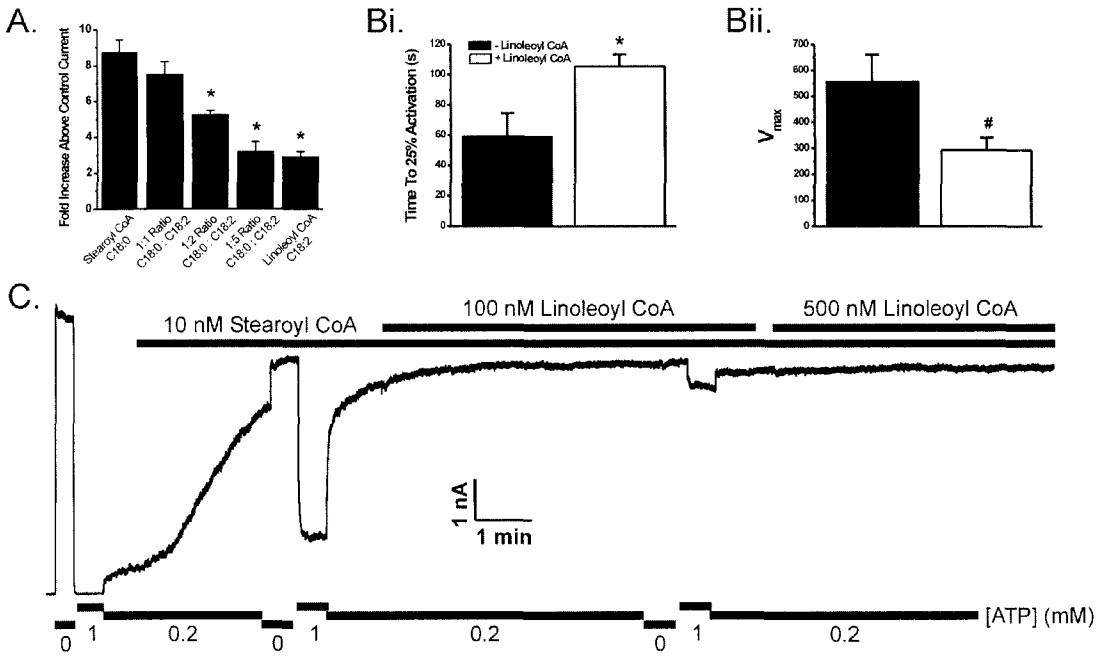
Trans acyl CoAs are equally effective activators of K_{ATP} channels relative to their *cis* acyl CoA counterparts. ***A*** and ***B.*** Representative wild-type Kir6.2/SUR1 (***A***) and Kir6.2(E23K/I337V)/SUR1 polymorphic (***B***) K_{ATP} channel recordings in response to 100 nM *cis*- ^{9}-oleoyl CoA and 100 nM *trans*-Δ^{9}-elaidoyl CoA. K_{ATP} channel currents were recorded as described previously (Figure 3-1). Dashed lines represent steady-state K_{ATP} channel current at 0.2 mM MgATP prior to and following application of the corresponding acyl CoA to the inside of the membrane patch. ***C.*** Compiled data detailing the effects of *cis* and *trans* long chain acyl CoAs on wild-type and E23K/I337V polymorphic K_{ATP} channel currents. $^{*}P < 0.05$ vs. wild-type counterpart (n = 5–8 patches per group).

Figure 3-3.



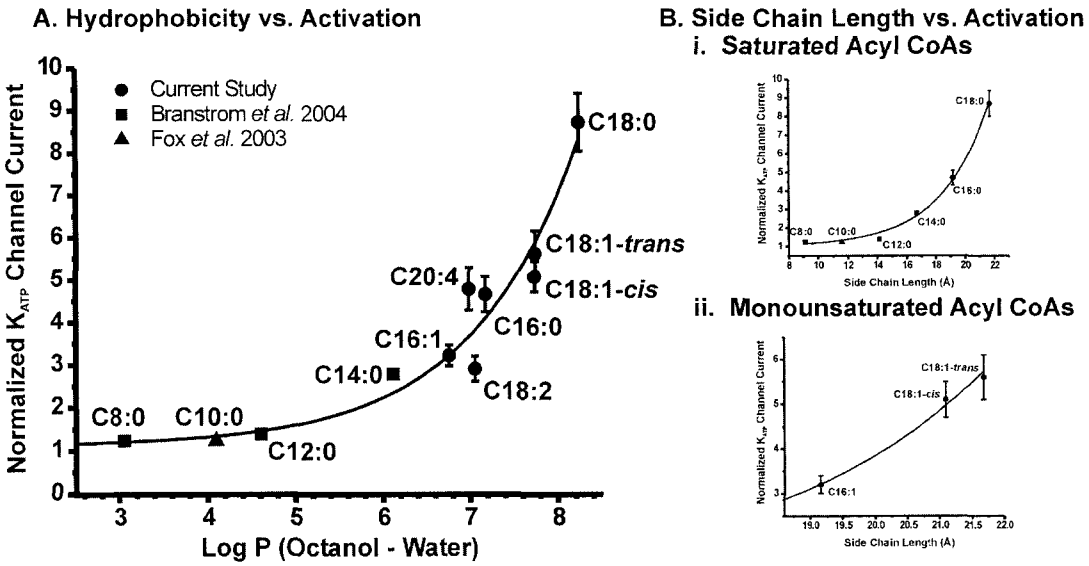
Fully saturated and *trans* acyl CoAs remain bound to the channel for a prolonged period of time unlike *cis* monounsaturated acyl CoAs. ***A*** and ***B***. Representative wild-type Kir6.2/SUR1 K_{ATP} channel recordings in response to 500 nM *cis*-Δ^9-oleoyl CoA (***A***) or *trans*-Δ^9-elaidoyl CoA (***B***). Black bars indicate the duration of acyl CoA stimulus. Each membrane patch was perfused for 5 minutes with control solution to allow washout of the acyl CoA. Black arrows denote level of K_{ATP} channel activity remaining following the 5 minute washout period. K_{ATP} channel currents were measured as described previously (Figure 3-1). ***C.*** Compiled data on the rate of activation for each acyl CoA. $\$P < 0.05$ vs. stearoyl CoA. ***D.*** Compiled data for rate of washout for each acyl CoA. Values in ***D*** were calculated as steady-state K_{ATP} channel current reached following the 5 minutes washout period over the maximum steady-state current attained during the acyl CoA stimulus and expressed as the mean ± SE. $*P < 0.05$ vs. oleoyl CoA at 100 nM; $\#P < 0.05$ vs. oleoyl CoA at 500 nM (n = 4–13 patches per group).

Figure 3-4.



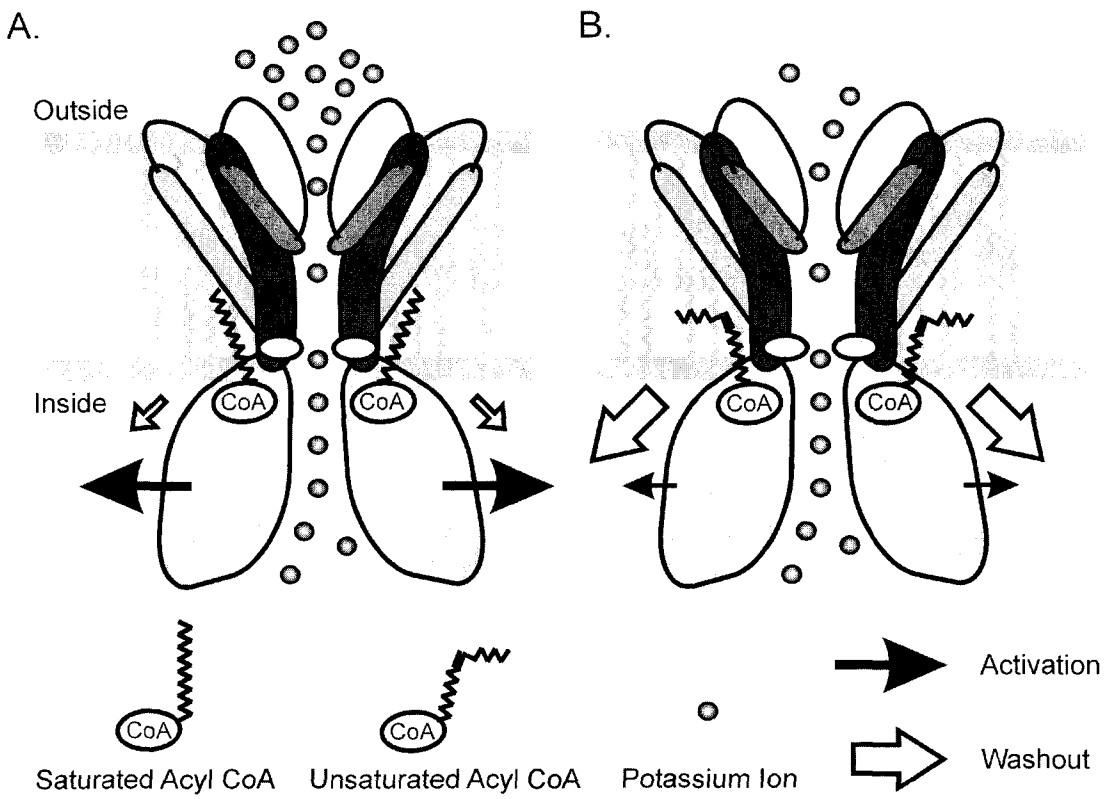
Unsaturated acyl CoAs reduce the efficacy of saturated acyl CoAs. ***A.*** Increasing the relative concentration of linoleoyl CoA decreases the ability of stearoyl CoA to activate the K_{ATP} channel. *$P < 0.05$ vs. stearoyl CoA. ***B.*** The response to 100 nM stearoyl CoA is both delayed (***Bi***) and slowed (***Bii***) when the membrane patch is pre-exposed to 500 nM linoleoyl CoA. V_{max} is defined as the maximum slope of activation measured during the acyl CoA stimulus. *,#$P < 0.05$ vs. membrane patches not pre-exposed to linoleoyl CoA (n = 6–9 patches per group). ***C.*** Activation by a low concentration of stearoyl CoA persists in the presence of saturating concentrations of linoleoyl CoA.

Figure 3-5.



Hydrophobicity (Log P) of acyl CoAs increases with increased acyl tail length and decreases with the introduction of double bonds. ***A.*** Data from the current study was plotted as the normalized current after application of 100 nM of the corresponding acyl CoA versus the partition coefficient between octanol and water. Data points were fitted to a single exponential using the equation $y = y_0 + Ae^{x/t}$. ***B.*** The correlation between acyl tail length and K_{ATP} channel activity is even stronger when the acyl CoAs are divided based on saturation (***i*** and ***ii***). Results from two previous studies were plotted as indicated (■ = (Branstrom et al., 2004); ▲= (Fox et al., 2003)). n = 6–14 patches per group.

Figure 3-6.



Schematic representation of the interaction of saturated (***A***) or unsaturated (***B***) acyl CoAs with the Kir6.2 subunit of the K_{ATP} channel and the plasma membrane. Saturated acyl CoAs activate the channel to a greater extent than unsaturated acyl CoAs but do not come unbound from the membrane as easily. Relative size of black and hollow arrows indicates relative change in K_{ATP} channel activity or acyl CoA washout respectively.

Table 3-1

				Fold Increase in Activity	
Name	**Designation**	**Sidechain length (Å)**	**Log P value**	**Wild type**	**E23K/I337V**
Stearoyl CoA	C18:0	21.70	8.23	8.7 ± 0.7	12.8 ± 1.6*
Elaidoyl CoA	C18:1 *trans-9*	21.68	7.73	5.6 ± 0.5	7.5 ± 0.4*
Oleoyl CoA	C18:1 *cis-9*	21.09	7.73	5.1 ± 0.4	8.1 ± 1.1*
Arachidonoyl CoA	C20:4 *cis-5,8,11,14*	24.14	6.98	4.8 ± 0.5	ND
Palmitoyl CoA	C16:0	19.18	7.17	4.7 ± 0.4	6.9 ± 0.6*
Palmitoleoyl CoA	C16:1 *cis-7*	19.16	6.75	3.2 ± 0.2	3.2 ± 0.4
Linoleoyl CoA	C18:2 *cis-9,12*	21.66	7.05	2.9 ± 0.3	2.1 ± 0.2
Myristoyl CoA	C14:0	16.66	6.11	ND (Branstrom et al., 2004)	ND
Lauroyl CoA	C12:0	14.14	4.60	ND (Branstrom et al., 2004)	ND
Decanoyl CoA	C10:0	11.62	4.09	ND (Fox et al., 2003)	ND
Octanoyl CoA	C8:0	9.10	3.05	ND (Branstrom et al., 2004)	ND

* indicates P<0.05 versus wild-type
ND = not determined in this study but see (ref)

Nomenclature and physical characteristics of each acyl CoA used in this study are shown. The relative effects on wild-type and E23K/I337V polymorphic β-cell K_{ATP} channels are detailed.

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Chapter 4

Metabolic Regulation of Cardiac Sodium/Calcium Exchange by Intracellular Acyl CoAs.

A version of this chapter has been submitted to EMBO Journal under the same title.

Introduction

Sodium calcium exchangers (NCX) are a class of membrane proteins intimately involved in the regulation of intracellular Ca^{2+} homeostasis, playing key roles in diverse signaling pathways in a variety of cell types. In the heart, the NCX1.1 isoform is a critical modulator of cardiomyocyte Ca^{2+} cycling, typically operating in forward-mode to extrude one Ca^{2+} ion for 3-4 Na^{+} ions (Dong et al., 2002; Hinata and Kimura, 2004) and generating an inward Na^{+} current that contributes to the plateau phase of the action potential (Weber et al., 2002). In addition to regulating physiological Ca^{2+} levels, alterations in the ionic, electrical and metabolic milieu that accompany cardiac pathologies such as ischemia/reperfusion (IR) injury (Schafer et al., 2001) and heart failure (Schillinger et al., 2003; Piacentino, III et al., 2002) can promote reverse-mode NCX1.1 activity leading to calcium overload and electrical dysfunction (Tani, 1990). In this regard, there is much interest in developing pharmacological inhibitors of reverse-mode NCX1.1 (Hobai and O'Rourke, 2004).

While much is known about the molecular biology and biophysics of NCX1.1, the intrinsic regulation of both forward- and reverse-mode NCX1.1 activity by endogenous signaling and metabolic pathways that may be altered in physiological and pathophysiological conditions has not been fully characterized. Identification of these regulatory processes may therefore provide valuable insights into the cellular mechanisms by which NCX1.1 is modulated in health and disease in a variety of tissues. One key area of interest is the role of lipids and lipid metabolism in regulating ionic homeostasis via modulation of ion channels and exchangers. Lipid-containing moieties such as the anionic phosphatidylinositol 4,5-bisphosphate (PIP_2) are capable of increasing

reverse-mode NCX1.1 activity by reducing Na^+-dependent (I_1) inactivation leading to a net increase in Ca^{2+} influx (Hilgemann and Ball, 1996; He et al., 2000). Interestingly, intracellular levels of anionic long chain fatty acyl CoA esters (acyl CoAs) are increased in heart tissue during exercise (Goodwin and Taegtmeyer, 2000), ischemia (Whitmer et al., 1978), hypertrophy (Finck et al., 2003) and in the failing heart (Sharma et al., 2004) due to changes in metabolic enzyme activity, substrate preference and oxygen supply. Acyl CoAs are akin to PIP_2 in that they also possess a negatively-charged head group (CoA) and a hydrophobic tail. Our group and others have shown that acyl CoAs are potent activators of ATP-sensitive potassium (K_{ATP}) channels (see chapters 2, 3 and Riedel et al., 2003; Larsson et al., 1996; Branstrom et al., 2004) and that acyl CoAs and PIP_2 may share a common mechanism of action (Schulze et al., 2003) via interaction with common positively-charged residues in the K_{ATP} channel (Manning Fox et al., 2004). Furthermore, we have shown that alterations in the sidechain length and degree of saturation of the acyl tail greatly affect the efficacy and persistence of K_{ATP} channel activation by acyl CoAs (see chapter 3 and Riedel and Light, 2005).

Therefore, we sought to determine 1) the effects of physiological levels of acyl CoAs with varying sidechain length and saturation on recombinant NCX1.1 activity and 2) the effects of intracellular acyl CoA elevation on NCX1.1-mediated Ca^{2+} overload in an intact cardiomyocyte model. Our findings provide the first direct evidence linking altered fat metabolism and lipotoxicity to NCX1.1 activity and have broad implications for our current understanding of NCX1.1 regulation and function in a variety of tissues.

Results

Reverse-mode NCX1.1 activity is increased by long chain acyl CoAs.

Previous measurements of electrogenic NCX1.1 currents have relied on either whole-cell (O'Rourke et al., 1999; Maack et al., 2005) or giant excised patch clamp techniques (Hilgemann, 1990; Matsuoka et al., 1997). Here we show that NCX1.1 current can be routinely measured using the conventional excised inside-out patch technique with minor modifications. Adenoviral infection of tsA201 cells with AdVNCX1.1 resulted in a >75% infection efficiency and excised inside-out membrane patches yielded average peak currents of 12.9 ± 1.1 pA. Reverse-mode NCX1.1 activity displayed a characteristic rapidly developed peak current followed by a slow Na^+-dependent (I_1) inactivation to a steady-state level that stabilized at 25 ± 2.2% of the peak current (Figure 4-1Ai; Hilgemann, 1990). NCX1.1 currents were sensitive to the NCX inhibitors $NiCl_2$ (5mM) and KB-R7943 (5μM), which resulted in a 91.1 ± 2.6% and 84.3 ± 4.9% inhibition of total current respectively, but were not inhibited by the selective L-type Ca^{2+} channel blocker nifedipine (Figure 4-1B).

The process of intracellular Na^+-induced I_1 inactivation (Hilgemann, 1990) is critically regulated by the 20 amino acid exchanger inhibitory peptide (XIP) sequence in the cytosolic portion of NCX1.1. Mutations throughout the XIP region dramatically altering inactivation properties (Matsuoka et al., 1997). In addition, PIP_2 has been shown to inhibit the transition of the active exchanger into its I_1 inactive state (He et al., 2000) therefore increasing reverse-mode activity. PIP_2 also influences the activity of a number of ion channels (Oliver et al., 2004; Suh and Hille, 2005) and in some cases shares a common mechanism of action with acyl CoAs (Schulze et al., 2003; Manning Fox et al.,

2004). In this study, we investigated the effects of acyl CoAs on NCX1.1 activity (Figure 4-1C,D). Application of 1μM palmitoyl CoA to the cytosolic surface of the membrane patch significantly inhibited reverse-mode I_1 inactivation (Figure 4-1C) resulting in a 214 ± 74% increase in the late-to-peak current ratio and a 65.2 ± 31.1% increase in total reverse-mode activity (AUC; Figure 4-2B,C). Conversely, we found that oleoyl CoA caused a small yet significant decrease in total forward-mode NCX1.1 activity (12.7 ± 4.3%; Figure 4-1D). As Ca^{2+}-loading via increased reverse-mode NCX1.1 is considered to be a major contributor to cardiac damage sustained during IR injury (Piper et al., 2003) we further examined the modulation of I_1 inactivation by acyl CoAs.

Regulation of I_1 inactivation by acyl CoAs demonstrates side chain length and saturation dependence.

Our group and others have previously shown that the regulation of K_{ATP} channels by acyl CoAs is dependent on both chain length and saturation (Riedel and Light, 2005; Branstrom et al., 2004). Here we tested a number of acyl CoAs on NCX1.1 activity and found similar trends to those observed for activation of the β-cell K_{ATP} channel (Figure 4-2; Riedel and Light, 2005). Specifically, we found that the shorter chain decanoyl CoA (C10:0) had no significant effect on either the late-to-peak current ratio or relative NCX1.1 activity (Figure 4-2A). Increasing the chain length to 16 carbons (palmitoyl CoA, C16:0) and further to 18 carbons (stearoyl CoA, C18:0) significantly increased both the late-to-peak current ratio (Figure 4-2B) and relative exchanger activity (Figure 4-2C). Addition of one double bond (oleoyl CoA, C18:1) caused an increase in NCX1.1 activity similar to that of palmitoyl and stearoyl CoA, while addition of a second double bond (linoleoyl CoA, C18:2) eliminated the effect of acyl CoAs on I_1 inactivation (Figure 4-

2B,C). Application of the n-3 polyunsaturated fish oil docosahexaenoyl (DHA) CoA resulted in a small yet significant decrease in total NCX1.1 reverse-mode activity resulting from a decrease in peak exchanger activity (Figure 4-2C).

Regulation of I_1 inactivation by acyl CoAs does not require ATP.

Several studies have demonstrated a stimulatory effect of Mg-ATP on NCX1.1 activity (Hilgemann and Ball, 1996; Hilgemann, 1990; Berberian et al., 1998; Condrescu et al., 1995), a mechanism which may occur via phosphorylation of PIP and the maintenance of membrane PIP_2 levels. To determine that the observed activation of NCX1.1 by acyl CoAs in our study was not contaminated by membrane-bound PIP_2 or by associated ATP-mediated events, we repeated our experiments in the absence of Mg-ATP (Figure 4-3). Under these conditions, I_1 inactivation occurred more rapidly ($\tau = 7.8 \pm 1.1$ sec vs. $\tau = 17.5 \pm 1.7$ sec; $P<0.01$) and to a greater extent than in the presence of 2mM Mg-ATP (Figure 4-3C, compare white bars). Perfusion of PIP_2-depleted membrane patches with acyl CoAs resulted in a much larger fold activation of NCX1.1 current (~3.8 and ~19 fold for palmitoyl and oleoyl CoA respectively) than in the presence of Mg-ATP (~2.4 fold for palmitoyl and oleoyl CoA, Figure 4-3C).

Acyl CoAs interact with XIP, but at a site distinct from that of PIP_2.

PIP_2 regulates NCX1.1 activity via interaction with the XIP region located on the intracellular loop between transmembrane segments 5 and 6 (Li et al., 1991) and residue F255 seems especially important in governing this PIP_2-mediated modulation (He et al., 2000). To assess whether acyl CoAs interact with NCX1.1 via a similar mechanism, we applied an antibody directed against XIP (AbXIP) directly to membrane patches. In the absence of additional modulators, AbXIP reduced peak current by ~80% while

eliminating the steady-state current. However, pre-exposing the membrane patch to 1μM oleoyl CoA resulted in a significantly impaired AbXIP-mediated inhibition of both peak and late currents (22.6 ± 8.2 and 32.5 ± 8.4 % inhibition respectively; Figure 4-4A). To elucidate further similarities between binding of acyl CoAs and PIP_2, we created the previously described F255E mutant (Matsuoka et al., 1997). NCX1.1(F255E) mutant exchangers inactivate more rapidly and PIP_2 is incapable of inducing NCX1.1(F255E) exchanger activity following I_1 inactivation (He et al., 2000) as a result of a weakened PIP_2-XIP interaction. Although we have successfully reproduced this finding using a similar protocol and found that acyl CoAs are likewise unable to stimulate significant activity in inactivated mutant NCX1.1 (Figure 4-4B), we report two additional observations. First, unlike PIP_2, oleoyl CoA can reduce inactivation when bound prior to NCX1.1(F255E) activation (Figure 4-4C). Under these conditions, total NCX1.1(F255E) activity was significantly increased 10.80 ± 3.34 fold ($P < 0.05$, n = 11) compared to a non-significant 3.09 ± 1.21 fold ($P = 0.11$, n = 11) when the exchanger was exposed to 30μM PIP_2. This suggests that the NCX1.1(F255E) mutant exchanger retains the ability to be significantly modified by acyl CoAs but not PIP_2. Secondly, we found that the inability of acyl CoAs to stimulate NCX1.1 activity in a population of inactivated transporters was a property limited to the F255E mutant. Figure 4-4D shows that oleoyl CoA readily stimulated wild-type NCX1.1 activity, increasing current levels by 91.6 ± 4.5 % (n = 5) over that measured at the base of the inactivation, while PIP_2 failed to do so. Conversely, even the wild-type exchanger was not significantly reactivated by PIP_2 (32.4 ± 14% increase in current following PIP_2 perfusion vs. base inactive current, n = 6 $P = 0.08$; Figure 4-4D).

Acyl CoA accumulation increases NCX1.1 reverse-mode activity in neonatal rat cardiomyocytes. Experiments from this section performed by Dr. István Baczkó.

Accumulation of long chain acyl CoAs within transgenic FACS-1 over-expressing mouse cardiomyocytes is associated with cardiomyopathy (Chiu et al., 2001), however the mechanisms underlying this association are not clearly delineated. In light of our own findings and those of Chiu and colleagues, we investigated the cellular consequences of acyl CoA accumulation and subsequent increases in NCX1.1 activity by examining Ca^{2+} loading via reverse-mode NCX1.1 activity in an intact model cellular system in which acyl CoA levels were manipulated. Rat neonatal cardiomyocytes were used for these experiments because of their robust expression of NCX1.1 (Boerth et al., 1994) and ability to maintain their phenotype under standard culture conditions. We used an established protocol for inducing reverse-mode NCX activity in intact spontaneously beating primary myocyte cultures that involves an initial loading of the cells with Na^+ via inhibition of the Na/K-ATPase pump using a K^+-free solution. Subsequent removal of extracellular Na^+ promotes Na^+ extrusion and concomitant Ca^{2+} loading via reverse-mode NCX1.1 activity (Figure 4-5A; Eigel and Hadley, 2001). Changes in cytosolic Ca^{2+} were monitored using the Ca^{2+}-sensitive fluorophore calcium green. The observed Ca^{2+} loading was nickel-sensitive but nifedipine insensitive and removal of external Ca^{2+} prevented Ca^{2+} loading (Figure 4-5E) – observations that are consistent with the measurement of reverse-mode NCX1.1 activity. Acyl CoA levels within neonatal rat cardiomyocytes were elevated by adenoviral-mediated over-expression of fatty acyl CoA synthetase-1 (FACS-1), the primary enzyme involved in FFA esterification to acyl CoAs (Figure 4-5C inset; Suzuki et al., 1990; Coleman et al., 2002). Incubation of

cardiomyocytes over-expressing FACS-1 in the presence of FFAs led to a significant increase in maximum and total Ca^{2+} loading (Figure 4-5B-D). To confirm the presence of elevated acyl CoA levels within the cardiomyocytes, we measured the total cytosolic acyl CoA content in each experimental group using HPLC (Figure 4-5F). Incubation with FFAs alone did not significantly increase total acyl CoA levels within the cells (0.039 ± 0.015 vs. 0.027 ± 0.01 pmoles/μg total protein in control cells). The expression of FACS-1 alone led to a non-significant increase in total acyl CoA content (0.171 ± 0.053 pmoles/μg total protein, $P = 0.14$). When FACS-1 treated myocytes were incubated with elevated FFAs, there was a significant ~7-fold increase in total acyl CoA content (0.174 ± 0.043 pmoles/μg total protein). It was in this treatment group that we observed the greatest activation of reverse-mode NCX1.1 activity and Ca^{2+} loading (Figure 4-5D).

Discussion

Accumulation of Ca^{2+} within the cardiomyocyte contributes to hallmark electrical and contractile dysfunction of IR injury (Carmeliet, 1999; Eigel and Hadley, 2001). The importance of reverse-mode NCX activity to Ca^{2+} overload in this setting is highlighted in a number of important studies. Of particular note is the observation that cardiac-specific over-expression of the NCX1.1 gene resulted in increased susceptibility to IR-mediated cardiac dysfunction (Cross et al., 1998), while antisense inhibition or complete cardiac-specific ablation of NCX1.1 provided significant protection (Eigel and Hadley, 2001; Imahashi et al., 2005). Increased NCX1.1 reverse-mode activity by whatever mechanism therefore represents a major burden to contractile recovery follow IR injury. Results from our study provide the first direct evidence that NCX1.1 activity is under the

influence of acyl CoAs. These common fatty acid intermediates represent a novel class of NCX1.1 modulators that may be involved in the observed increase in NCX1.1 reverse-mode activity in IR injury as well as the physiological coupling of Ca^{2+} homeostasis to lipid metabolism in a variety of tissues.

Mechanistic Insights

Previous studies have demonstrated that transition of active reverse-mode NCX1.1 to the inactive I_1 state is governed by intracellular sodium ions and the endogenous regulatory XIP sequence (Hilgemann et al., 1992; Matsuoka et al., 1997). Our results provide evidence that acyl CoAs may interact with NCX1.1 at or near this XIP sequence as these anionic lipids can prevent an XIP antibody from reaching its epitope. We also show that acyl CoAs may disrupt the XIP-mediated conformational change required for NCX1.1 to inactivate thereby increasing Ca^{2+} loading under conditions that stimulate reverse-mode NCX1.1 activity.

The differences observed between the effects of acyl CoAs on the wild-type and F255E mutant exchanger provide insights into the molecular mechanisms mediating XIP-induced inactivation. While wild-type NCX1.1 can be prevented from entering the I_1 inactive state in the presence of both PIP_2 (He et al., 2000) and acyl CoAs (this study), wild-type NCX1.1 can be re-activated only by acyl CoAs. This suggests that the ability of the endogenous XIP region to mediate inactivation is hindered by acyl CoAs. Addition of a negative charge in the XIP region of the NCX1.1(F255E) mutant may act to repel the negatively charged PIP_2 and acyl CoA molecules resulting in the observed enhancement of inactivation (Figure 4-4B,C). Differences in either net charge or charge location may explain why PIP_2 is less capable of altering NCX1.1(F255E) activity than

acyl CoAs (Figure 4-4C). The inability of either PIP_2 or acyl CoAs to reactivate mutant NCX1.1(F255E) currents (Figure 4-4B) suggest that the interaction of XIP with these anionic lipids is weakened by the addition of a negative charge at position 255 of the XIP region facilitating a faster rate of inactivation.

Physiological Significance

Long chain acyl CoAs are dynamically regulated within cardiomyocytes and serve as a major substrate for β-oxidation and lipogenesis. Acyl CoAs are increased in both physiological [during exercise (Goodwin and Taegtmeyer, 2000)] and pathophysiological [during IR (Whitmer et al., 1978)] situations and are known regulators of a number of proteins including ion channels (Haber et al., 2003; Faergeman and Knudsen, 1997). The total acyl CoA pool in the heart is large but free cytosolic acyl CoA levels are highly buffered to levels near 1μM by several acyl CoA and fatty acid binding proteins (Faergeman and Knudsen, 1997). While actual free acyl CoA levels at distinct sub-cellular locations are not known, it has been shown that acyl CoA levels increase during cardiac ischemia (Whitmer et al., 1978) as plasma fatty acid levels increase. Accordingly, we show that acyl CoAs (at 1μM) have a dual effect on NCX1.1 activity, slightly inhibiting forward-mode activity while dramatically increasing reverse-mode activity. During exercise, when the exchanger is operating primarily in forward-mode to extrude Ca^{2+}, a small decrease in NCX1.1 activity may have a positive inotropic effect by maintaining higher or prolonged intracellular Ca^{2+} levels and may decrease the likelihood of arrhythmogenic delayed afterdepolarizations by shortening the action potential (Bers et al., 2002). Conversely, under pathophysiological conditions such as IR injury where reverse-mode activity is favored, a large increase in reverse-mode NCX1.1

activity by acyl CoAs may result in increased Ca^{2+} loading thus further impairing contractile function and increasing the likelihood of developing Ca^{2+}-dependent pro-arrhythmic events. In this regard, reverse-mode NCX1.1 inhibitors have been shown to reduce the occurrence of ischemia-induced arrhythmias (Takahashi et al., 2003).

The contribution of elevated plasma FFAs to the severity of IR injury is not clearly established in humans, however it has been shown that obese adult Zucker Fatty rats exhibit greater contractile dysfunction following a period of global ischemia than their lean control counterparts (Sidell et al., 2002; Zhou et al., 2000). This contractile dysfunction was completely reversed with the use of PPARγ agonists that among other effects, decrease plasma FFA levels (Sidell et al., 2002; Zhou et al., 2000) suggesting that elevated FFAs may increase the risk for contractile dysfunction in the ischemic heart. These results support our suggestion that increased reverse-mode NCX activity under conditions of elevated intracellular saturated long chain acyl CoAs represents an important novel regulatory pathway contributing to cardiac dysfunction in multiple pathological settings including ischemia, hypertrophy, diabetic cardiomyopathies, heart failure and other conditions associated with lipotoxic heart disease.

Acyl CoA Saturation and Implications for Ischemia/Reperfusion Injury

Altering the composition of dietary fatty acids can change cellular membrane constituents and alter lipid profiles within cells (Hulbert et al., 2005). Modulation of both the level and composition of intracellular acyl CoAs that fluctuate as a result of changing metabolism may serve as an important modulatory mechanism for controlling signaling processes including NCX1.1 activity. We demonstrate that modulation of NCX1.1 by acyl CoAs is dependent on the number of carbons and double bonds present in the

hydrophobic tail with longer chain more saturated acyl CoAs causing the greatest increases in reverse-mode NCX1.1 activity. In particular, we have shown that saturated and monounsaturated acyl CoAs of chain length 16 or greater are more effective NCX1.1 activators than shorter chain or polyunsaturated acyl CoAs. We have established a similar dependence on side-chain length and degree of saturation for the activation of K_{ATP} channels by acyl CoAs (see chapter 3 and Riedel and Light, 2005). This suggests that the severity of contractile dysfunction sustained during IR injury may be linked to plasma FFA composition and hence to the type of fat consumed in the diet with saturated fats preferentially increasing susceptibility to IR injury via enhanced reverse-mode NCX1.1 activity. The potential importance of our findings is supported by previous studies that show diabetic mice fed diets high in long chain fatty acids exhibited increased susceptibility to cardiomyopathies while those fed diets high in medium chain fatty acids did not (Finck et al., 2003). In this respect, we show that the medium chain decanoyl CoA was incapable of modulating NCX1.1 activity. Moreover, the inhibitory effects of omega-3 fish oils on cardiac sodium channels and NCX1.1 have been documented (Leaf and Xiao, 2001; Xiao et al., 2004). Xiao and colleagues have shown that the polyunsaturated fats DHA and eicosapentaenioc acid (EPA) inhibit both forward and reverse-mode NCX activity whereas the saturated stearic acid does not (Xiao et al., 2004). Accordingly, we show that the fish oil ester DHA CoA inhibits reverse-mode NCX1.1 activity. Therefore, the combined actions of non-esterified and esterified fish oils on NCX1.1 activity may contribute to the well-documented cardioprotective effects of dietary fish oils (Kris-Etherton et al., 2002; Marchioli et al., 2002). Conversely, diets higher in saturated fats such as stearic or palmitic acid may increase susceptibility to IR

injury via enhanced reverse-mode NCX1.1 activation. Taken together, these results may therefore provide a contributory mechanism for the observed benefits of strategies designed to lower serum lipids and/or specifically decrease long chain fatty acyl triglycerides in the treatment of diabetic cardiomyopathies (Finck et al., 2003).

Implications for Other Tissues

NCX is expressed in a number of tissues including the heart, endocrine pancreas, and brain. Three separate genes have been identified, each encoding several splice variants in these and other tissues (Quednau et al., 1997) suggesting that NCX activity is an important physiological process for the maintenance of appropriate ion homeostasis. NCX1 is the most ubiquitously expressed of the three genes and alternatively spliced variants are highly homologous, with differences from one splice variant to another existing only within a small alternative splicing region in the large intracellular loop (Quednau et al., 1997). The XIP region, which we have identified as a putative acyl CoA interaction site, is conserved among all NCX1 isoforms (Quednau et al., 1997) and exhibits between 65 and 70% sequence homology in NCX2 and NCX3 respectively (Li et al., 1994; Nicoll et al., 1996). The presence of the XIP region in all NCX gene products suggests that modulation of NCX by acyl CoAs may serve as an important Ca^{2+} regulatory mechanism in many tissues. This may be especially important in disease states such as obesity and diabetes, where lipid metabolism can be altered by high levels of plasma free fatty acids (Golay et al., 1986; Reaven et al., 1988) leading to elevated intracellular acyl CoA levels. To this extent, the following chapter details preliminary work examining the interaction of acyl CoAs with an abundantly expressed pancreatic β-cell NCX1 isoform, NCX1.3.

In conclusion, our results provide evidence for a novel link between cellular metabolism and calcium homeostasis. Given that free acyl CoA levels within cardiomyocytes are dynamically regulated according to metabolic demand as well as during several pathologies, we speculate that acyl CoAs may be a key endogenous regulator of NCX1.1 activity in the heart. Further studies are therefore warranted to determine the contributions of acyl CoA modulation of NCX activity to cellular function both under physiological and pathophysiological conditions in a variety of tissues expressing this important class of ion exchanger.

Research Design and Methods

Molecular Biology. The rat heart NCX1.1 (Low et al., 1993) clone in pcDNA3 was obtained from Dr. Jonathan Lytton and the NCX1.1 adenovirus obtained from Dr. Joseph Cheung. Labeling of amino acids beginning with the first in-frame methionine results in the highly conserved XIP region spanning amino acids 251 to 270. Therefore, the equivalent mutation of F223 from previous studies (He et al., 2000; Matsuoka et al., 1997) corresponded to F255 in our clone. The discrepancy occurs due to the potential existence of an NH_2-terminal 32 amino acid signaling sequence that may be cleaved prior to insertion into the plasma membrane (Nicoll et al., 1990). To generate the F255E XIP region mutant, the NCX1.1 construct was used to generate an F255E fragment using Proofstart DNA polymerase (Qiagen, Mississauga, ON). The primers used to generate the F255E fragment were F255E upper: 5'-AGGCGGCTTCTCGAGTAACAAGTAT-3' and F255E lower: 5'-GACATACTTGTACTCGAGAAGCCG-3'. This amplified fragment was then digested and cloned into NCX1.1 using the specific endonucleases

AgeI and NsiI. The presence of the mutation was verified by diagnostic digest and sequence analysis.

Cell Culture. tsA201 cells [an SV40-transformed variant of the HEK293 human embryonic kidney cell line that is devoid of endogenous NCX activity (Figure 4-1Ai)] were maintained in DMEM supplemented with 25 mM glucose, 2 mM L-glutamine, 10% FCS, and 0.1% penicillin/streptomycin in a humidified incubator at 37°C with 5% CO_2. Cells were passaged and plated at 60 – 80% confluency in 35 mm culture dishes ~8 hours prior to infection or transfection.

Isolation of Neonatal Rat Cardiac Myocytes. Neonatal cardiac myocytes were isolated by Suzanne Kovacic in Dr. Jason Dyck's laboratory from the hearts of 1- to 3-day-old neonatal rat pups according to a protocol approved by the University of Alberta Animal Policy and Welfare Committee and conforming to the Canadian Council on Animal Care (CCAC) Guidelines, as described previously (Kovacic et al., 2003). The cells were plated on fibronectin coated coverslips at ~100% confluency.

Cellular Infection/Transfection. Wild-type NCX1.1 was expressed in tsA201 cells via adenovirus delivery of the NCX1.1 and green fluorescent protein clones (GFP; pGreenLantern, Life Technologies, Gaithersburg, MD) driven under separate CMV promoters. tsA201 cells were exposed to ~30 pfu/cell AdVNCX1.1 for 2 – 4 hours and macroscopic NCX1.1 current recordings were performed 24 – 48 hours later.

The NCX1.1(F255E) mutant exchanger was inserted into the mammalian plasmid vector pcDNA3.1 and introduced to tsA201 cells using the Ca^{2+} phosphate precipitation technique as described in previously (see Chapter 2). Transfected tsA201 cells were identified using fluorescent optics in combination with co-expression of GFP.

Macroscopic NCX1.1(F255E) recordings were performed 24 – 48 hours after transfection.

After 18 h of culture at 37°C in 5% CO_2, neonatal rat cardiomyocytes were allocated to 3 different groups. Group 1 cells were continued in culture with no additional treatment. Group 2 and 3 cells were infected with 10 pfu/cell AdVFACS-1. Twenty-four hours post-infection, group 3 cells were incubated with 0.2 mM palmitate and 0.1 mM oleate complexed to BSA in DMEM/F12 media for 18 h.

Electrophysiology. The excised inside-out patch clamp technique was used to measure macroscopic outward (reverse-mode) and inward (forward-mode) NCX currents from infected or transfected tsA201 cells. Large tip diameter patch pipettes were pulled from borosilicate glass (G85150T-3; Warner Instruments Inc., Hamden CT) to yield resistances between 400 and 700 KΩ when backfilled with buffer solution. Once a GΩ seal was formed, the membrane patch was excised from the cell and positioned in the path of a multi-input perfusion pipette.

For outward reverse-mode NCX current measurements, the pipette (extracellular) solution contained the following (in mM): CsCl 140, TEA 20, HEPES 5, glucose 10, $MgCl_2$ 1.4, and $CaCl_2$ 4. pH was adjusted to 7.4 with CsOH. Outward currents were elicited by rapidly switching from an intracellular cesium-based solution containing (in mM): CsCl 120, TEA 20, HEPES 5, glucose 10, $MgCl_2$ 1.4, and $CaCl_2$ 4.28 to an intracellular sodium-based solution containing (in mM): CsCl 30, NaCl 90, TEA 20, HEPES 5, glucose 10, $MgCl_2$ 1.4, and $CaCl_2$ 4.28. Free calcium concentrations of these intracellular solutions were buffered to ~800 nM with 5 mM EGTA and pH was adjusted to 7.2 with CsOH.

For inward forward-mode NCX current measurements, the pipette (extracellular) solution contained (in mM): CsCl 30, NaCl 90, TEA 20, HEPES 5, glucose 10, $MgCl_2$ 1.4, and $CaCl_2$ 4.28. Free calcium concentrations were buffered to ~800 nM with 5 mM EGTA and pH was adjusted to 7.4 with CsOH. Inward currents were elicited by rapidly changing the intracellular solution from a cesium-based low calcium solution containing (in mM): CsCl 120, TEA 20, HEPES 5, glucose 10, $MgCl_2$ 1.4, $CaCl_2$ 4.28, and EGTA 5, to a cesium-based high calcium solution containing (in mM): CsCl 140, TEA 20, HEPES 5, glucose 10, $MgCl_2$ 1.4, and $CaCl_2$ 4. pH of the intracellular solutions was adjusted to 7.2 with CsOH. In some experiments, 2 mM MgATP was added to both extracellular and intracellular solutions where indicated in the text. All solution changes were achieved in <2 sec.

The holding potential of the membrane patch was 0 mV and the elicited NCX currents were measured and analyzed using an Axopatch 200B amplifier and Clampex 8.1 software (Axon Instruments, Foster City, CA). All experiments were performed at room temperature (22 ± 1°C).

Measurement of Ca^{2+} transients. Neonatal rat cardiomyocytes from three different groups outline above were rinsed and loaded for 30 min at room temperature and for 30 min at 37°C with the Ca^{2+}-sensitive fluorescent probe Calcium Green-1AM (4 μM, in a 1:1 v/v dimethyl sulfoxide:pluronic acid mixture; Molecular Probes, Eugene, OR, USA). After loading, cells were washed and the coverslips were placed on an inverted microscope (Olympus, CK40) for observation at 220X. A Photomultiplier Detection System (PTI, Lawrenceville, NJ, USA) with Clampex software (version 8.1) was used for data acquisition and analysis. Calcium Green-1AM was excited with 480 nm light and

the emitted light intensity at 520 nm was digitized and stored. Cells were subjected to a superfusion protocol designed to evoke reverse-mode NCX activity as described previously (Eigel and Hadley, 2001). Briefly, cells were superfused for 2 min with a solution containing (in mM): NaCl 140, KCl 4, HEPES 10, $CaCl_2$ 2.5, $MgCl_2$ 1, glucose 10 to evoke control Ca^{2+} transients. Cells were then superfused for 5 min with a K^+-free solution containing (in mM): NaCl 144, HEPES 10, $CaCl_2$ 2.5, $MgCl_2$ 1, glucose 10 that resulted in intracellular Na^+ loading. Finally, a Na^+-free solution containing (in mM): LiCl 140, KCl 4, HEPES 10, $CaCl_2$ 2.5, $MgCl_2$ 1, glucose 10 was superfused for 5 min to evoke reverse-mode NCX activity. pH was adjusted to 7.4 in all solutions. TTX (20 μM) was added to the K^+-free and Na^+-free solutions to block TTX-sensitive I_{Na}, thus preventing Na^+ loading via voltage-gated Na^+ channel activity. $NiCl_2$ (5 mM) was added to the Na^+-free solution in some experiments to inhibit NCX activity (Hinde et al., 1999). The increase in diastolic Ca^{2+} indicator fluorescence and the area under the curve during reverse-mode NCX activity were normalized to the amplitude of the Ca^{2+} transient under control conditions. The Ca^{2+} transient experiments were performed at room temperature (22 ± 1°C).

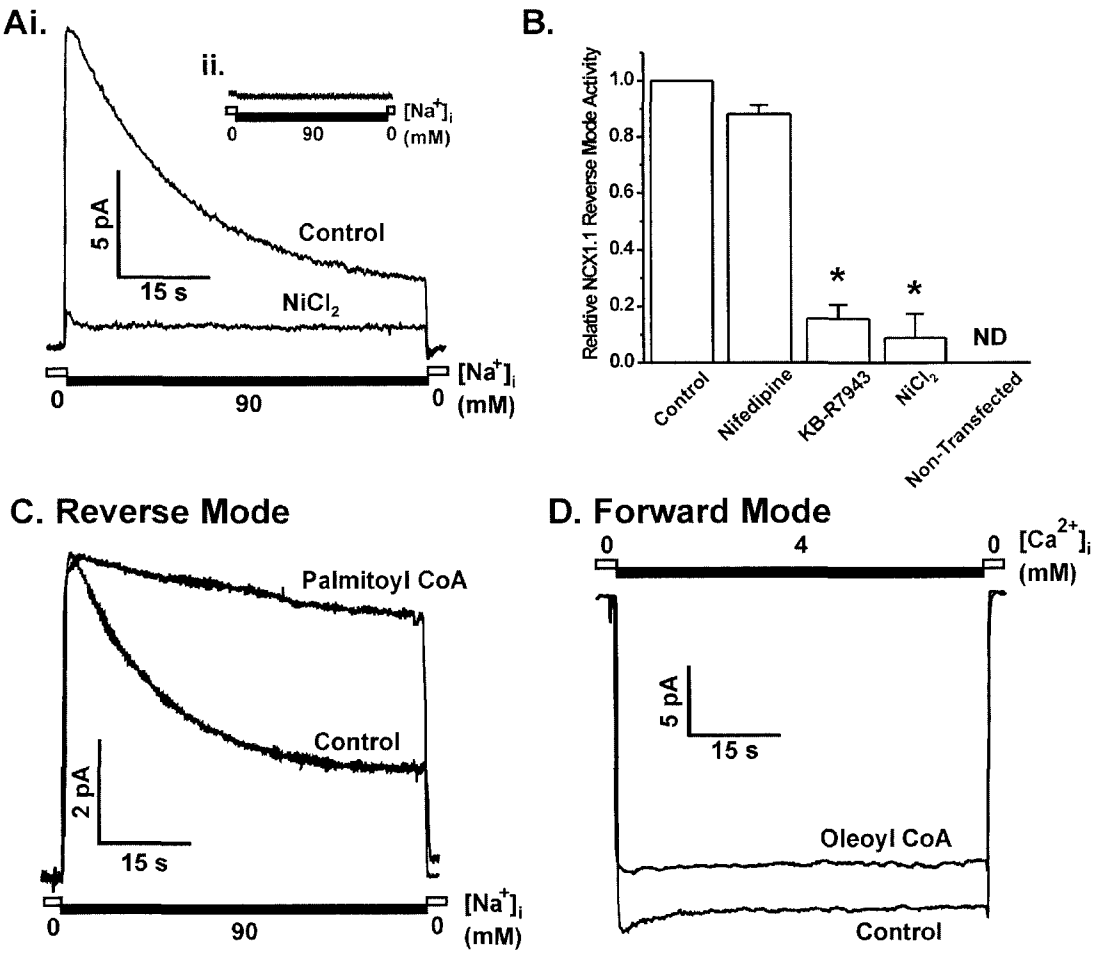
Experimental Compounds. Nifedipine was dissolved in DMSO as a 10 mM stock solution and diluted to concentrations indicated in text prior to use. The final DMSO concentration of 0.1% did not affect NCX currents in the absence of nifedipine (data not shown). $NiCl_2$ was dissolved directly into solutions to concentrations indicated in the text. All long chain acyl CoAs were purchased from Sigma (Sigma-Aldrich, Oakville, ON) as Li^+ salts and dissolved in ddH_2O as 10 mM stock solutions. PIP_2 was purchased from Avanti Polar Lipids Inc. (Alabaster, AL) as a tri-ammonium salt and dissolved in

ddH_2O as a 5 mM stock solution. Prior to use, stock solutions were sonicated for 5-10 min and diluted in intracellular solution to concentrations indicated in the text. The α-XIP antibody (Alpha Diagnostic Intl., San Antonio, TX) was reconstituted in PBS at 1 mg/ml, and subsequently diluted 1:100 in intracellular solution prior to use.

Determination of Free Acyl CoA Content. The free intracellular acyl CoA content of neonatal myocytes was determined by Ken Strynadka using HPLC as described previously (Larson and Graham, 2001). Cells were plated at 100% confluency on 60mm culture dishes and treated as described in text with fatty acids and/or AdVFACS-1 virus. Values obtained were normalized to total protein content of the cells lysed from each dish.

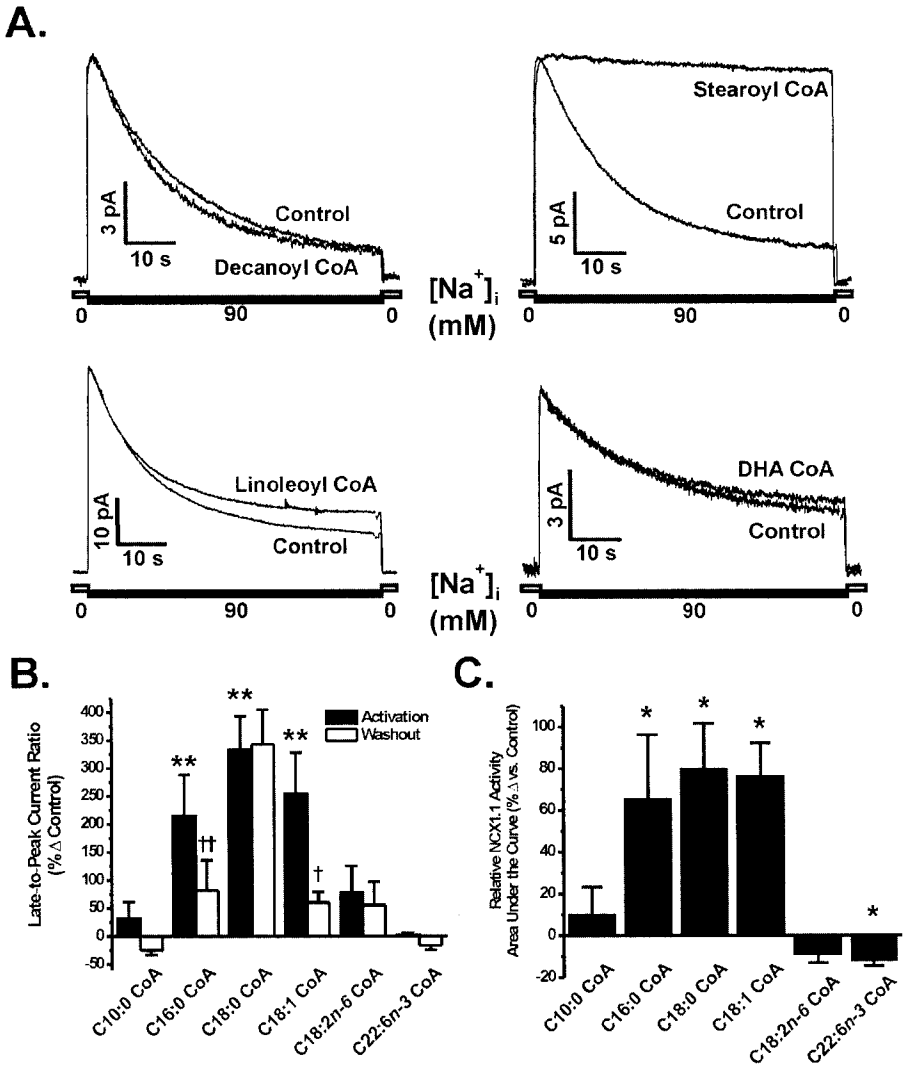
Statistical Analysis. Recombinant macroscopic NCX1.1 currents were normalized to the stable peak current obtained under control conditions. Inactivation was calculated as the ratio of the steady-state current obtained after 1 minute of activation to the peak current obtained during activation (late-to-peak current ratio). Total NCX1.1 current was measured as the area under the activation curve during the 1 minute activation period. Statistical significance was assessed using the paired or unpaired student's *t* test where required. $P < 0.05$ was considered significantly different. Data are expressed as means ± SE.

Figure 4-1.



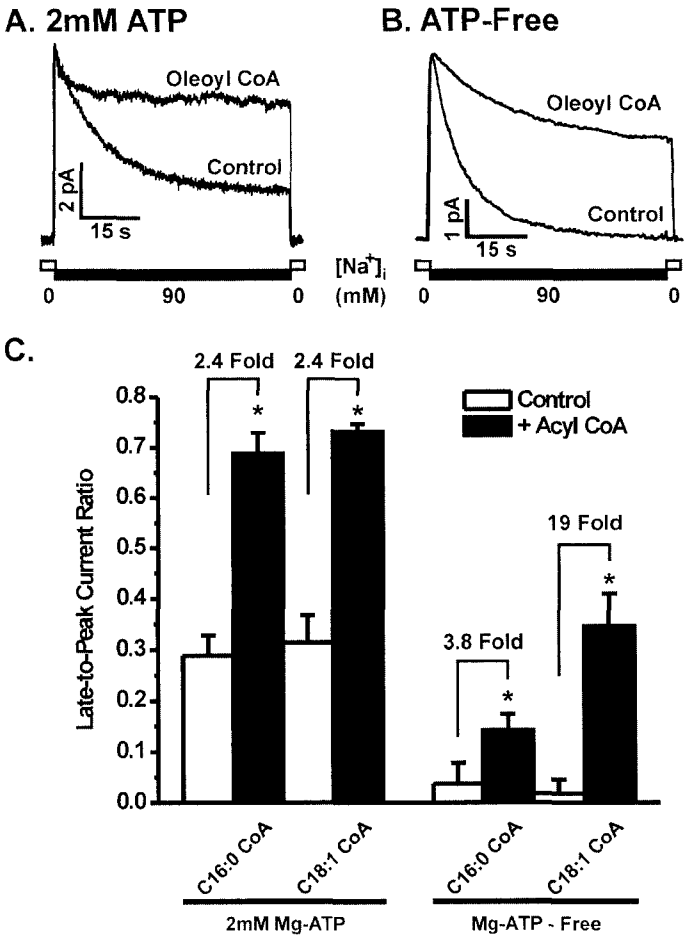
Forward and reverse-mode NCX1.1 activity is modulated by long chain acyl CoA esters. ***Ai.*** Representative macroscopic current recording of reverse-mode NCX1.1 activity measured in tsA201 cells following infection with AdVNCX1.1 virus showing activation by high intracellular Na^+ and inhibition by $NiCl_2$ (5 mM). ***Aii.*** Uninfected tsA201 cells are devoid of endogenous NCX activity. ***B.*** Grouped data showing inhibition of reverse-mode NCX1.1 activity by the specific inhibitors KB-R7943 (5 μM) and $NiCl_2$ (5 mM) but not by the L-type Ca^{2+} block nifedipine (10 μM). n=3-6 patches per group, $*P<0.05$ versus control, ND=not detected. ***C.*** Representative recording showing that palmitoyl CoA (1 μM) inhibits I_1 inactivation, thus increasing NCX activity. ***D.*** Representative current recording indicating that oleoyl CoA (1 μM) inhibits forward-mode NCX1.1 activity.

Figure 4-2.



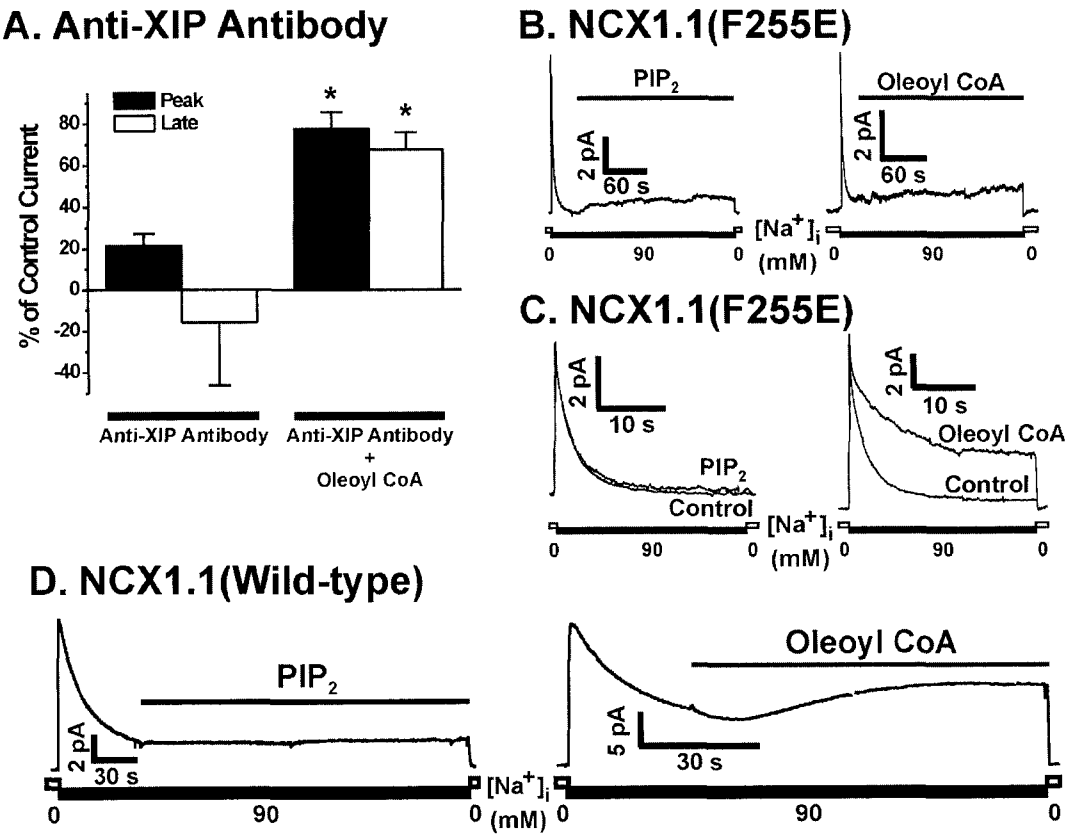
Activation of reverse-mode NCX1.1 changes with acyl CoA saturation and chain length. ***A.*** Representative macroscopic NCX1.1 current recordings showing that short chain (decanoyl CoA, C10:0) and polyunsaturated acyl CoAs (linoleoyl CoA, C18:2 and DHA CoA, C22:6) do not inhibit I_1 inactivation, unlike stearoyl CoA (C18:0). ***B.*** Grouped data showing the maximum effect (black bars) and reversibility (white bars) of each acyl CoA on the late-to-peak current ratio (I_{late}/I_{peak}). n=3-11 patches per group. $^{**}P<0.01$ versus control I_{late}/I_{peak} in respective group, $^{\dagger}P<0.05$ and $^{\dagger\dagger}P<0.01$ versus maximum activation in respective group. ***C.*** Grouped data indicating total NCX1.1 reverse-mode activity was increased in the presence of palmitoyl, stearoyl, and oleoyl CoA only. n=4-6 patches per group. $^{*}P<0.05$ versus control activity measured in the same patch prior to acyl CoA application.

Figure 4-3.



Activation of reverse-mode NCX1.1 activity by acyl CoAs occurs in the absence of Mg-ATP. Representative macroscopic NCX1.1 current recordings in the presence (*A*) and absence (*B*) of 2mM Mg-ATP. Under both conditions, acyl CoAs (oleoyl CoA, 1 μM shown here) inhibited I_1 inactivation. ***C.*** Grouped data indicating that the fold increase in late-to-peak current ratio as a result of acyl CoA exposure (black bars) was greater in the absence of Mg-ATP. n=7-11 patches per group. **P*<0.05 vs. corresponding control patches in the absence of acyl CoAs (white bars).

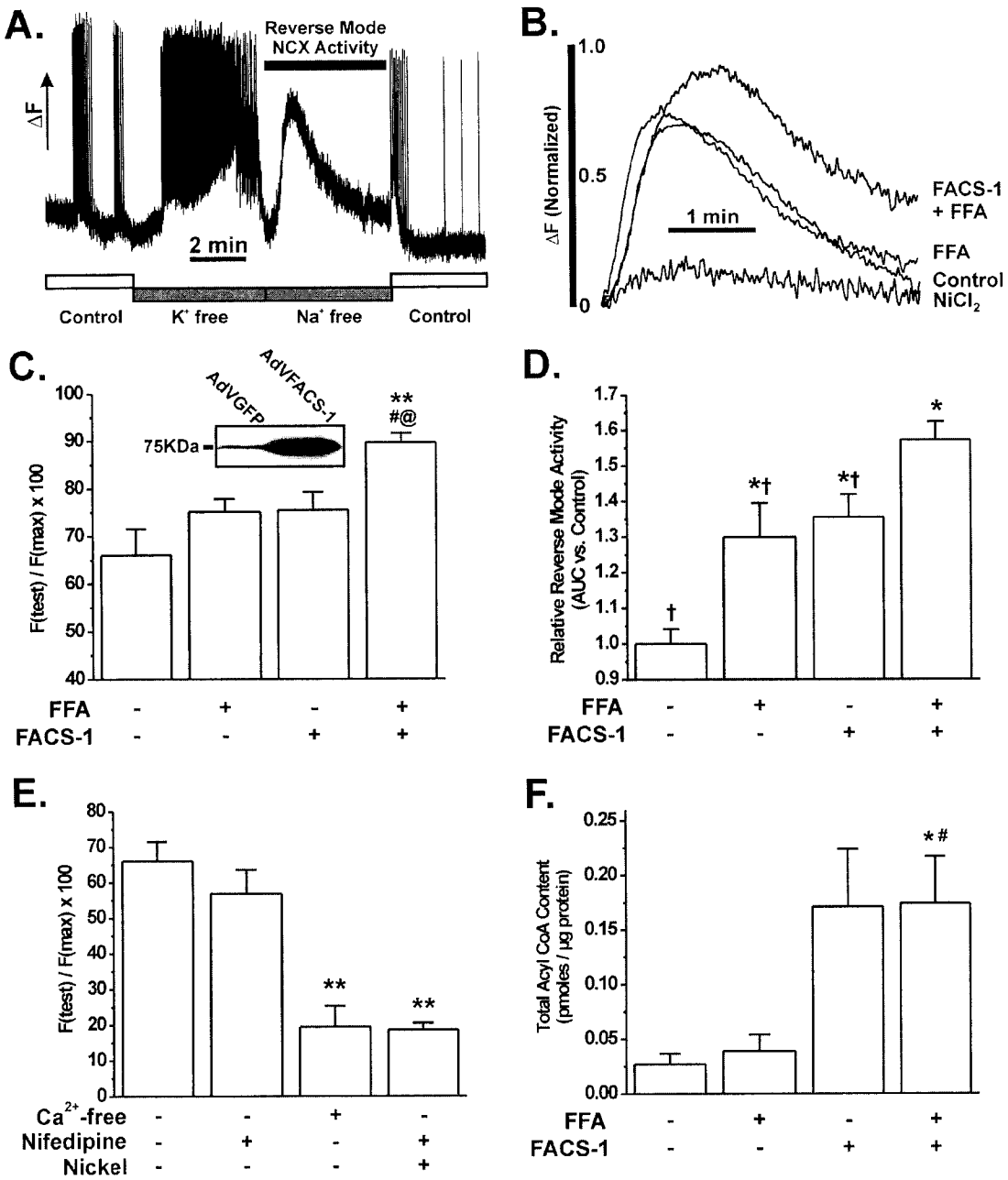
Figure 4-4.



The activation of reverse-mode NCX1.1 activity by long chain acyl CoA esters may occur via their interaction with the XIP region, but at a site distinct from that of PIP_2. ***A.*** Grouped data indicating that pre-exposure of membrane patches to oleoyl CoA (1 μM) abolishes the inhibitory action of an anti-XIP antibody (1:100 dilution). n=3-4 patches per group. *$P<0.05$ versus respective peak (black bars) and late (white bars) current in patches exposed to the anti-XIP antibody alone. ***B.*** Representative current recording indicating that neither acyl CoAs (1 μM oleoyl CoA shown here) or PIP_2 (30 μM) are able to reactivate NCX1.1(F255E) mutant exchanges following I_1 inactivation. ***C.*** Representative current recordings indicating that acyl CoAs (1 μM oleoyl CoA shown here) but not PIP_2 (30 μM) are able to prevent the entry of NCX1.1(F255E) mutant exchangers into the I_1 inactive state. ***D.*** Representative macroscopic current recording indicating the ability of acyl CoAs (oleoyl CoA, 1 μM shown here) but not PIP_2 (30 μM) to reactivate wild-type NCX1.1 exchanger activity following a period of I_1 inactivation.

Reverse-mode NCX1.1 activity is increased in neonatal rat cardiomyocytes following elevation of intracellular acyl CoA levels. ***A.*** Representative Ca^{2+} transient recording from a group of neonatal rat cardiomyocytes (6-8 cells) subjected to a protocol designed to induce reverse-mode NCX1.1 activity. ***B.*** Representative recordings showing typical reverse-mode NCX1.1-mediated Ca^{2+} loading in neonatal cardiomyocytes. Panel shows only the reverse-mode NCX1.1 activity-related portion of the recording obtained during Na^{+}-free solution exposure. ***C.*** Grouped data showing significant increase in maximum Ca^{2+} loading in FACS-1 over-expressing neonatal cardiomyocytes incubated with 0.2 mM palmitate and 0.1 mM oleate. n=5-7 experiments per group with 6-8 cells per experiment. $^{**}P<0.01$ vs. control group, $^{\#}P<0.05$ vs. FFA group, $^{@}P<0.05$ vs. FACS-1 group. **Inset:** Representative western blot showing over-expression of FACS-1 protein after treatment with AdVFACS-1 virus. ***D.*** Total reverse-mode NCX1.1 activity was increased by either elevated fatty acids or FACS-1 over-expression. FACS-1 over-expressing cells incubated with fatty acids showed significantly increased total NCX1.1 activity versus non fat-incubated over-expressers. n=5-7 experiments per group with 6-8 cells per experiment. $^{*}P<0.05$ vs. control group. $\dagger P<0.05$ vs. FFA + FACS-1 group. ***E.*** Total reverse-mode NCX1.1 activity was inhibited by a Ca^{2+}-free extracellular perfusate and by the specific inhibitor $NiCl_2$ (5 mM) but not by the L-type Ca^{2+} channel blocker nifedipine (10 μM). n=3-4 experiments per group with 6 to 8 cells per experiment. $^{**}P<0.01$ vs. control group. ***F.*** Total cytosolic acyl CoA levels were increased significantly by palmitate and oleate incubation in FACS-1 over-expressing cells. n=3 experiments per group. $^{*}P<0.05$ vs. control group. $^{\#}P<0.05$ vs. FFA group.

Figure 4-5.



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Chapter 5

Metabolic Regulation of β-Cell Sodium/Calcium Exchange by Intracellular Acyl CoAs.

Introduction

In the pancreatic β-cell, metabolism of glucose leads to an oscillatory electrical activity that regulates insulin secretion in a pulsatile manner (Henquin and Meissner, 1984; Ashcroft and Rorsman, 1989). These oscillations in membrane potential are mirrored by similar frequency oscillations in the intracellular calcium concentration (Gilon and Henquin, 1992). Thus, regulation of calcium entry and calcium removal from the β-cell is a critical process regulating the appropriate secretion of insulin in response to nutrient stimuli.

The regulation of β-cell calcium levels involves a complex interplay of several calcium transport mechanisms between a number of calcium pools. Evidence suggests that both the inositol 1,4,5-trisphosphate (IP_3) and ryanodine receptors may be present in β-cells, contributing to calcium-induced calcium release (CICR; Hagar and Ehrlich, 2000; Islam, 2002). However it is currently unclear what role CICR may have in initiating or maintaining insulin secretion. The observation that extracellular calcium removal abolishes glucose-stimulated insulin secretion (Hughes et al., 1987) suggests that entry of calcium via plasma membrane calcium channels represents a key physiological process. The current accepted hypothesis for the regulation of this K_{ATP} channel-dependent calcium influx is as follows. Metabolism of glucose in the β-cell results in the production of ATP at the expense of ADP. This rise in the ATP-to-ADP ratio closes the K_{ATP} channel, thus decreasing the membrane potassium permeability causing membrane depolarization. Once this depolarization reaches a threshold potential near -40mV, L-type calcium channels are activated resulting in a transient influx of calcium that triggers

the exocytosis of insulin-containing granules. The rise in membrane potential and intracellular calcium then triggers the activation of voltage-gated and calcium-activated potassium channels that serve to repolarize the β-cell membrane thus re-establishing the resting membrane potential (Ashcroft and Rorsman, 1989).

The critical processes governing the re-establishment of basal intracellular calcium levels are less well understood. β-cells express a host of intracellular and plasma membrane-bound proteins that regulate uptake of calcium into intracellular stores or extrusion of calcium to the extracellular space. These include the endoplasmic reticulum and plasma membrane calcium ATPases, mitochondrial calcium pumps, and the plasma membrane-associated sodium/calcium exchanger (NCX; Chen et al., 2003). While the presence of NCX in β-cells was proposed nearly forty years ago (Hales and Milner, 1968) its contribution to normal β-cell function has yet to be determined. In fact, the relative contribution of this exchanger to calcium influx versus calcium removal has not been definitively determined.

In addition to modulating intracellular calcium levels, NCX activity will also produce a net positive current in the direction of sodium flow. This results from the exchange of 3-4 sodium ions for every one calcium ion transported (Dong et al., 2002; Hinata and Kimura, 2004). As a result of this electrogenic stoichiometry and similar to ion channels, exchanger activity is influenced by both electrical and chemical gradients. However, unlike ion channels that are typically influenced by the concentration gradient of a single ion, the exchanger is influenced by the chemical gradient of both sodium and calcium. As the concentrations of these ions change in the β-cell along with the

membrane potential (electrical gradient), the direction of sodium flow can be reversed. Several studies have attempted to identify the predominant mode of NCX operation in β-cells (Gall and Susa, 1999; Gall et al., 1999; Van Eylen et al., 1998; Van Eylen et al., 2002). It is thought that under both resting and stimulating conditions, the β-cell NCX is likely to operate in forward mode, acting primarily as a calcium extrusion mechanism. There is some speculation suggesting that at the peak of calcium-driven action potentials where the β-cell membrane potential can reach positive potentials, the exchanger may be capable of operating in reverse mode, bringing calcium into the cell (Van Eylen et al., 1998). A physiological role for reverse mode NCX activity under these circumstances has not yet been demonstrated but may involve calcium-induced calcium release.

Nevertheless, the importance of β-cell NCX activity is highlighted in studies where NCX activity has been either artificially increased through over-expression (Van Eylen et al., 2002) or decreased using antisense oligonucleotides (Van Eylen et al., 1998). These studies indicate that the oscillatory nature of β-cell electrical activity is influenced by NCX. Therefore, the identification of potential endogenous modulators of β-cell NCX activity may be of critical importance. One potential class of endogenous modulator of interest to our group is long chain acyl CoAs. We have previously shown that these intracellular fatty acid metabolites are potent modulators of β-cell K_{ATP} channel activity and may influence the activity of β-cells especially in the settings of obesity and type 2 diabetes where levels of intracellular acyl CoAs are elevated (See chapters 2, 3, and Riedel et al., 2003; Riedel and Light, 2005). We have therefore also begun to examine the effects of long chain acyl CoAs on β-cell NCX activity.

Results

NCX1.3 forward mode activity is increased by acyl CoAs

Studying the sodium/calcium exchanger in intact tissues such as pancreatic β-cells remains difficult due to the lack of specific inhibitors as well as the relatively small currents that are generated by its activity. We have used a model mammalian cell system in which the predominant β-cell isoform NCX1.3 is over-expressed to study the modulation of current by acyl CoAs. Measurement of NCX1.3 activity from transiently transfected tsA201 cells was performed using the classical excised inside-out patch clamp technique with minor modifications. Using the calcium phosphate precipitation transfection technique, we achieved >50% transfection efficiency using co-expression of GFP with NCX1.3 (Figure 4-1A inset). Activation of the NCX1.3 isoform using a high internal calcium concentration yielded average peak currents of 21.2 ± 1.8 pA. As described above (See Chapter 1) and unlike NCX1.1, activation of forward mode NCX1.3 resulted in a rapid peak followed by a slow inactivation to a steady-state on average of 68.7 ± 1.9% of the peak current. The measured current was subject to ~50% inhibition by the NCX specific inhibitor KB-R7943 (5 μM) that was reversible upon washout (Figure 5-1A,D). Although KB-R7943 was originally suggested to be reverse-mode specific (Iwamoto et al., 1996), more recent evidence suggests that forward-mode activity is also susceptible to KB-R7943 inhibition (Birinyi et al., 2005). However, this is the first known test of this compound on the NCX1.3 isoform and thus the previous noted mode-specificity of KB-R7943 may not be applicable here.

Application of 1 μM palmitoyl CoA to the internal face of excised membrane patches resulted in a decrease in NCX1.3 inactivation that manifested itself as an increase

in the late-to-peak current ratio from 0.697 ± 0.04 under control conditions to 0.865 ± 0.02 in the presence of palmitoyl CoA (Figure 5-1B,C). This reduction in inactivation led to a significant increase in total NCX1.3 forward-mode activity of 12.7 ± 2.8% (Figure 5-2D).

Acyl CoAs exhibit chain length and saturation dependence

We have previously shown that modulation of the β-cell K_{ATP} channel by acyl CoAs exhibits dependence on both the side chain length and degree of saturation (see Chapter 3 and Riedel and Light, 2005). Here we tested the hypothesis that a similar relationship exists between acyl CoAs and NCX1.3 (Figure 5-2). Application of the medium chain decanoyl CoA (C10:0) resulted in no significant modulation of either peak current or inactivation (Figure 5-2A,F,G). Increasing the chain length to 16 carbons (palmitoyl CoA, C16:0) resulted in the above mentioned increase in total NCX activity. A further increase in chain length by 2 carbons (stearoyl CoA, C18:0) resulted in a large reduction in inactivation (late-to-peak current ratio of 0.71 ± 0.02 in control vs. 0.85 ± 0.02 with stearoyl CoA; Figure 5-2B,G). In the absence of a significant change in peak current, this inhibition of inactivation caused a significant increase in total NCX activity of 16.6 ± 2.0% (Figure 5-2H). The introduction of one double bond (oleoyl CoA, C18:1) yielded a significant increase in peak current (Figure 5-2C,F) and maintained the effect on inactivation (Figure 5-2G) resulting a large increase in total NCX1.3 activity of 35.7 ± 7.9% (Figure 5-2H). The addition of a second double bond (linoleoyl CoA, C18:2) eliminated the effect of the acyl CoA on both the peak current and the inactivation process (Figure 5-2D and F-H), producing a slight but non-significant reduction in total NCX1.3 activity. Finally, we tested the effect of the polyunsaturated omega-3 fish oil

ester, docosahexaenoyl (DHA) CoA on NCX1.3 activity. The hydrophobic tail of this acyl CoA is comprised of 22 carbons with 6 double bonds. As with linoleoyl CoA, the other polyunsaturated acyl CoA used in this study, DHA CoA had non-significant effects on each of the parameters tested here (Figure 5-2E-H).

Acyl CoAs can reverse forward mode inactivation of NCX1.3

The molecular mechanisms governing the observed inactivation of forward mode NCX1.3 activity are unknown. It is unclear at this time whether this process is regulated by the binding of sodium or calcium ions to their respective sites on the internal or external side of the membrane or whether it involves a mechanism similar to the so-called "ball-and-chain" inactivation that occurs in a number of ion channels where a portion of the protein itself is responsible for inhibiting the transport of ions across the membrane (Armstrong and Bezanilla, 1977). In either case, application of 1 μM oleoyl CoA to a population of NCX1.3 that had already undergone inactivation resulted in a reactivation of activity (Figure 5-3). The level of activity reached at steady-state (85 ± 2.5 % of control peak current) was not significantly different from the initial peak current recorded prior to inactivation nor was it significantly different from the peak current obtained following a brief 2 minute inactivation of current in the presence of oleoyl CoA (Figure 5-3B, compare points 3 and 4). Preliminary results suggest that a similar effect occurs in the presence of the fully saturated stearoyl CoA.

Discussion

Despite the obvious importance of calcium flux in the process of insulin secretion, much research remains to be performed before a complete understanding of the processes that regulate intracellular calcium levels can be obtained. In fact, the relative

contribution of extracellular calcium and intracellular calcium stores to the rise in cytosolic calcium that precedes insulin secretion is not known (Tamarina et al., 2005), nor is it known whether it is a local or global rise in calcium that is responsible insulin release (Henquin et al., 1998; Mears, 2004). It is generally accepted however that the activation of voltage-gated calcium channels and the subsequent influx of calcium is important at least for the release of insulin-containing granules in the immediately releasable pool (IRP). Recent studies suggest that L-type calcium channels may physically interact with the secretory machinery, thus stimulating exocytosis of these granules and generating the observed first phase of insulin secretion (Mears, 2004). If this is the case, smaller increases in cytosolic calcium could be sufficient to raise local calcium concentrations above the threshold for granule exocytosis. If only a small rise in calcium is required to trigger exocytosis, there must be mechanisms in place to ensure that cytosolic calcium levels can be rapidly returned to normal. One likely mechanism is the sodium/calcium exchanger. Herchuelz and colleagues have shown that β-cell NCX activity may account for up to 70% of the calcium removal machinery (Van Eylen et al., 1998). In this report, we show that long chain acyl CoAs, which are dynamically regulated in the β-cell during both normal metabolism and in pathologies including obesity and type 2 diabetes, are capable of increasing NCX1.3 activity by up to 36%. This is the first report to demonstrate that β-cell NCX activity is regulated by lipids and lipid metabolism and may yield an additional mechanism by which accumulation of long chain acyl CoAs could be diabetogenic.

Mechanistic insights

The process of Na^+-dependent inactivation in reverse-mode cardiac NCX1.1 operation has been well studied and is detailed elsewhere in this thesis (See Chapter 1 and Chapter 4). As this is the first report detailing direct measurement of NCX1.3 currents, this is also the first report to observe this forward-mode inactivation process. Therefore, the mechanisms underlying the observed inactivation that takes place during forward-mode NCX1.3 activity remain to be elucidated. This lack of knowledge stems largely from the fact that NCX activity has been mainly studied in cardiomyocytes where expression of NCX1.3 is absent. Future studies are warranted to address this issue.

Interestingly, there is a high sequence identity between the cardiac NCX1.1 isoform and the β-cell NCX1.3 isoform (Quednau et al., 1997). The differences exist within a small alternatively spliced region in the intracellular loop between transmembrane domains 5 and 6. Within this splicing region is a cassette of between 2 and 5 exons totaling up to 76 amino acids. The combination of expressed exons determines the splice variant that is created. NCX1.1 contains exons A, C, D, E, and F while NCX1.3 contains exons B and D. The total number of amino acids that differ between these two isoforms is 58. This is a relatively small area that could easily be investigated for such differences as phosphorylation consensus sequences (Ruknudin et al., 2000) and ion or other molecule binding regions.

Does β-cell NCX activity mediate calcium uptake or extrusion?

The stoichiometry of NCX is 3 or 4 Na^+ to 1 Ca^{2+}. The direction of flow of sodium is dependent on the concentration gradient for sodium and calcium as well as the membrane potential. The regulation of these three factors is a dynamic process. The

intracellular calcium and sodium concentrations and the membrane potential are in constant flux as metabolism oscillates in the β-cell. The extracellular concentrations of sodium and calcium are likely constant or relatively constant during the process of β-cell stimulation but may undergo alterations on a longer time scale due to changes in factors such as diet and exercise rates or during certain pathologies. In the β-cell, it has been shown that stimulation with high concentrations of glucose will lower the intracellular sodium concentration from ~14 mM at rest (3 mM glucose) to ~11 mM in a β-cell stimulated with 22 mM glucose (Saha and Grapengiesser, 1995). At the same time, free calcium concentrations in active β-cells oscillate between a basal concentration of near 150 nM to anywhere between 300 nM to near 1 μM (Van Eylen et al., 1998). As these ion concentrations change, so does the dependence of the NCX on membrane potential. For example, under resting conditions were the intracellular concentrations of sodium and calcium are approximately 14 mM and 150 nM respectively, the reversal potential for the NCX is calculated to be approximately -68 mV (see equation 1-1). This number represents the membrane potential at which the exchanger will exhibit no net flow of charge in either direction. At potentials more positive to -68 mV, the exchanger will extrude sodium and import calcium, thus operating in reverse-mode. At potentials more negative to -68 mV, which includes the resting membrane potential of a β-cell (on the order of -75 to -85 mV), the exchanger will operate in forward-mode, extruding calcium and bringing sodium into the cell. When a β-cell is stimulated by glucose, the intracellular sodium concentration decreases (Saha and Grapengiesser, 1995), the intracellular calcium concentration increases, and the membrane depolarizes (Ashcroft

and Rorsman, 1989). Examining the reversal potential of NCX1.3 under these circumstances ($[Na^+]_i$ = 11 mM; $[Ca^{2+}]_i$ = 1 μM), we find that the exchanger will reverse at potentials more positive to -2 mV. Interestingly, in a properly functioning β-cell, the membrane potential rarely exceeds this highly depolarized potential and if so, this only occurs at the peak of the calcium-driven action potential. While good experimental evidence is lacking to support these calculations at this time, the general trend of the exchanger operating primarily in forward mode has been demonstrated by others. In fact the evidence that does exist for the exchanger to contribute to the inflow of calcium (reverse-mode) comes from studies in which β-cells were exposed to 50 mM K^+ to depolarize the membrane (Van Eylen et al., 1998). Although not directly measured by Van Eylen and colleagues, this high concentration of K^+ would likely produce a large depolarization that would not only activate L-type calcium channels but could also shift the exchanger into reverse mode. Without a concomitant decrease in the intracellular sodium concentration, it is possible that the relationship between membrane potential and exchanger mode of operation would not be sufficiently shallowed to prevent the flipping of the exchanger into reverse mode. 50 mM K^+ does not necessarily represent a physiological depolarization and calls into question whether the exchanger could be capable of operating in reverse mode under physiological conditions.

Physiological Significance

In this study we observe a 10 – 35% increase in total forward-mode NCX1.3 activity in the presence of acyl CoAs with longer chain saturated and monounsaturated acyl CoAs having the greatest effect. Although the effects of increased forward-mode NCX activity were not measured on intact β-cells, we speculate that this increase in

activity may affect β-cell electrical activity and insulin secretion. First, because it is generally accepted that the exchanger operates predominantly in the forward-mode as a calcium extrusion mechanism, we hypothesize that such an increase in NCX activity may serve to inhibit or limit the normal rise in intracellular calcium required to initiate exocytosis. This initial first phase of insulin secretion occurs as a result of calcium-dependent exocytosis of insulin granules in the immediately releasable pool. It has been suggested that granules in the IRP display a range of calcium sensitivities and are therefore released at different rates depending on the calcium concentration reached during β-cell stimulation (Straub and Sharp, 2004). Therefore, this first phase of insulin secretion may be reduced as a result of increased forward-mode NCX activity and reduced intracellular calcium concentrations. However, it must be noted that recent work has suggested that L-type calcium channels physically interact with the IRP (Mears, 2004), and as such, localized calcium concentrations may be higher than the global calcium concentration. In this case, it is possible that a slightly more rapid extrusion of the calcium that enters via L-type calcium channels may not alter this first phase of granule exocytosis. There is currently no evidence that NCX interacts with physically with either the L-type calcium channel or the IRP. Such an interaction may be of physiological importance and studies are warranted to address this issue. It is possible that even in the absence of such an interaction, an inhibition of global increases in intracellular calcium via increased forward mode NCX1.3 activity could prevent or delay the second phase of insulin secretion. This phase is thought to occur via several mechanisms, some of which involve a global rise in intracellular calcium levels. These mechanisms include the recruitment of additional granules from the reserve pool to the

IRP (Rorsman and Renstrom, 2003) as well as activation of calcium-dependent amplifying pathways (Henquin, 2000). A reduction in this global calcium rise may therefore inhibit second phase insulin secretion. Coupled with the previously described increase in K_{ATP} channel activity in the presence of acyl CoAs (See chapters 2 and 3), we can speculate that the increase in calcium extrusion that would occur during increased forward-mode NCX activity may act to further reduce the insulin secretion response to glucose, possibly contributing to the onset of type-2 diabetes. This remains to be tested experimentally.

Secondly, is it possible that increased forward-mode NCX1.3 activity could actually stimulate insulin secretion? This may seem counter-intuitive; however the exchanger is electrogenic and when operating in forward-mode, will generate a net inward current. Any such inward current will depolarize the β-cell membrane, making it more likely to reach the threshold required to activate L-type calcium channels and thus potentially triggering insulin release. This mechanism has not been directly tested and therefore additional experiments are warranted. It has been shown however that antisense oligonucleotides-mediated knockdown of NCX activity in intact β-cells results in a decreased glucose-induced rise in intracellular calcium (Van Eylen et al., 1998). While the authors argue that this points to an importance of reverse-mode NCX activity in regulating β-cell function, an alternative mechanism may be in operation. Inhibition of forward-mode NCX activity may remove an additional depolarizing influence on the β-cell, making it more difficult for the β-cell to maintain a sufficiently depolarized membrane potential to activate L-type calcium channels. Therefore, the response to

glucose in this case may be reduced or delayed. Both a reduction and delay in the response to 11.1 mM glucose was observed following antisense NCX knock down (Van Eylen et al., 1998). Conversely, an acyl CoA-mediated increase in forward-mode NCX activity could potentially increase this depolarizing current, thereby aiding in the maintenance of the threshold potential and promoting insulin secretion. In this case, the effects of elevated acyl CoAs on NCX1.3 activity could be viewed as a compensation mechanism in an attempt to overcome the hyperpolarizing influence of these same acyl CoAs on the activity of K_{ATP} channels (see Chapters 2, 3 and Riedel et al., 2003; Riedel and Light, 2005). While purely speculative at this point, it is possible to test these mechanisms through specific inhibition and activation of NCX activity in the β-cell using the XIP peptide or over-expression of the protein itself. It must be acknowledged however that in the presence of elevated acyl CoAs, increased activity of the K_{ATP} channel will result in a hyperpolarization of the plasma membrane (see chapters 2 and 3). Under these circumstances, a larger depolarizing current would be required for the membrane potential to reach the threshold for calcium channel activation. Therefore, it is unclear whether the depolarizing influence of increased forward-mode NCX activity could be sufficient to initiate insulin secretion in the presence of elevated acyl CoAs and enhanced K_{ATP} channel activity.

Previous studies using over-expression of NCX1.7 in a β-cell insulinoma model cell line suggest however that the likely role of forward-mode NCX activity is to limit the rise in calcium (Van Eylen et al., 2002). This is expected as the exchanger operates as a calcium extrusion mechanism in forward-mode. However, were the depolarizing influence of forward-mode activity to result in increased insulin secretion, this would

have to occur as a result of increased transient influxes of calcium through L-type calcium channels. Van Eylen and colleagues (2002) have shown that over-expression of NCX1.7 results in a reduced intracellular calcium rise as measured by fura-2 fluorescence. Interestingly, it has been reported that increasing NCX activity in a mathematical model of β-cell electrical activity prolonged the plateau fraction (See Chapter 2) most likely due to the depolarizing influence of the inward generated Na^+ current during forward-mode operation (Gall and Susa, 1999). Two important points must be noted here. First, the work by Van Eylen et al. (2002) uses over-expression of the NCX1.7 isoform. The difference between NCX1.3 and NCX1.7 is the addition of exon F, 23 amino acids that are absent in the NCX1.3 isoform (Quednau et al., 1997). It is possible therefore that the biophysical characteristics of these two isoforms differ in such a way that over-expression of NCX1.3 may yield different effects on β-cell calcium handling. Secondly, the work of Gall et al. primarily uses mathematical modeling to examine the role of NCX activity on β-cell electrical activity (Gall and Susa, 1999). Experimentally, the validity of the model is not tested under physiological conditions. Instead, extracellular sodium reduction is used to examine the effect of reverse-mode NCX activity on β-cell membrane potential (Gall et al., 1999). Under these conditions, a reduction in the burst duration is noted, as would be expected if the predominant role of reverse-mode NCX activity was to repolarize the membrane. Interestingly, in this same study, NCX-mediated tail currents were observed during depolarizing/repolarizing voltage steps from -80 mV to 0 mV (Gall et al., 1999). These tail currents were inward in direction suggesting that the exchanger operates in forward-mode under these conditions. More physiological experiments are warranted to re-address the issue of whether the

depolarizing influence of the inward current or the intracellular calcium reducing properties of forward-mode NCX activity dominate the effect on β-cell electrical activity and insulin secretion.

We do not currently know if the effects of acyl CoAs on β-cell NCX activity alter β-cell excitability. It will be important in the future to assess the potential consequences of the observed 10 – 36% increase in total NCX1.3 activity on primary β-cell excitability. However, small changes in the activity of other ion channels [including K_{ATP} channels, which are 99% closed under resting conditions (Cook et al., 1988)] can lead to significant changes in β-cell excitability and dramatic effects on insulin secretion. This is due mainly to the very high input resistance of the β-cell in the presence of glucose. Over-expression of NCX1.7 (~2 fold increase in function) dramatically altered the effect of 17 mM glucose on intracellular calcium (Van Eylen et al., 2002) suggesting that increases in forward-mode NCX activity by acyl CoAs could potentially affect β-cell function.

Summary

In conclusion, we have provided the first evidence of metabolic regulation of β-cell NCX activity and suggest that a link may exist between elevated acyl CoAs and NCX activity that may alter β-cell electrical activity. Given that intracellular acyl CoA levels are dynamically regulated in a number of physiological and pathophysiological settings, we speculate that these fatty acid metabolites might serve an important role in regulating intracellular β-cell calcium homeostasis. Further research is required to uncover the physiological role that NCX plays in the secretion of insulin, especially

under conditions such as obesity and type 2 diabetes, where elevated acyl CoA levels have been observed and previously described as diabetogenic.

Research Design and Methods

Molecular Biology. The rat NCX1.3 isoform subcloned into pcDNA3 was kindly provided by Dr. Lytton at the University of Calgary, Alberta (Dunn et al., 2002).

Cell Culture and Transfection. tsA201 cells (an SV-40-transformed variant of the HEK293 human embryonic kidney cell line) were maintained in DMEM supplemented with 25 mM glucose, 2 mM L-glutamine, 10% FCS, and 0.1% penicillin/streptomycin in a humidified incubator at 37°C with 5% CO_2. Cells were passaged and plated at 60 – 80% confluency on 35-mm culture dishes ~4 hours prior to transfection. The NCX1.3 clone was then transfected into the tsA201 cells using the calcium phosphate technique. Transfected cells were identified using fluorescent optics in combination with co-expression of the green fluorescent protein plasmid (pGreenLantern; Life Technologies, Gaithersburg, MD). Macroscopic NCX1.3 current measurements were then performed 48 – 72 hours after transfection.

Electrophysiology. The excised inside-out patch clamp technique was used to measure macroscopic inward (forward mode) NCX1.3 currents from transfected tsA201 cells. Large tip diameter patch pipettes were pulled from borosilicate glass (G85150T-3; Warner Instruments Inc., Hamden, CT) to yield resistances between 400 and 900 KΩ when backfilled with buffer solution. Once a GΩ seal was formed, the membrane patch was excised from the cell and positioned in the path of a multi-input perfusion pipette.

For inward (forward mode) NCX1.3 current measurements, the pipette (extracellular) solution contained the following (in mM): CsCl 30, NaCl 90, TEA-Cl 20,

HEPES 5, Glucose 10, MgATP 2, $MgCl_2$ 1.4, and $CaCl_2$ 4.28. The free calcium concentration was buffered to ~1 μM with 5 mM EGTA. pH was adjusted to 7.4 with CsOH. Inward currents were elicited by rapidly switching from an intracellular low calcium solution containing (in mM): CsCl 120, TEA-Cl 20, HEPES 5, Glucose 10, MgATP 2, MgCl2 1.4, CaCl2 4.28 and EGTA 5, again yielding a free calcium concentration of ~1 μM, to a high calcium solution containing (in mM): CsCl 140, TEA-Cl 20, HEPES 5, Glucose 10, MgATP 2, $MgCl_2$ 1.4, and $CaCl_2$ 4. The final calcium concentration of this solution was calculated as ~3.2 mM using the sliders program (v1.00) provided free online by Dr. C. Patton (www.stanford.edu/~cpatton/maxc.html). pH of these intracellular solutions were adjusted to 7.2 using CsOH.

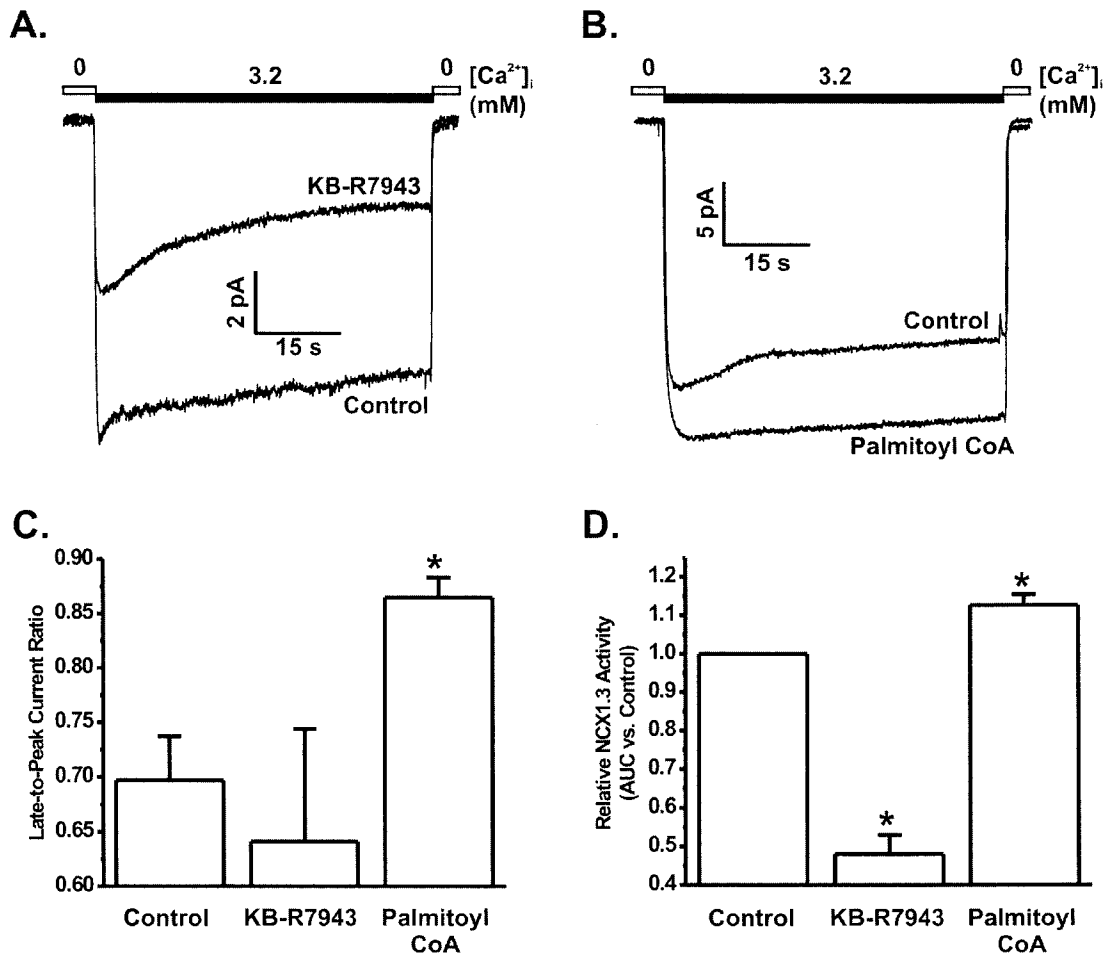
The membrane patch was held at a potential of 0 mV and the elicited NCX1.3 currents were measured and analyzed using the Axopatch 200B amplifier and Clampex 8.0 software (Axon Instruments, Foster City, CA). All experiments were performed at room temperature (22 ± 1°C).

Experimental Compounds. Long chain acyl CoAs decanoyl CoA (C10:0), palmitoyl CoA (C16:0), stearoyl CoA (C18:0), oleoyl CoA (C18:1), and linoleoyl CoA (C18:2), were purchased from Sigma-aldrich (Oakville, ON) as Li^+ salts and dissolved in ddH2O as 10 mM stocks. Docosahexaenoyl CoA (C22:6) was purchased from Avanti Polar Lipids (Alabaster, AL) in chloroform. Prior to use, stock solutions were sonicated for 5 – 10 minutes and diluted in intracellular solutions to concentrations indicated in the text.

Statistical Analysis. Recombinant macroscopic NCX1.3 currents were normalized to the stable peak current obtained under control conditions. Inactivation was calculated as the ratio of the steady-state current obtained after 1 minute of activation to the peak current

obtained during activation (late-to-peak current ratio). Total NCX1.3 current was measured as the area under the activation curve during the 1 minute activation period. Statistical significance was assessed using the paired or unpaired student's *t* test where required. $P < 0.05$ was considered significantly different. Data are expressed as means ± SE.

Figure 5-1.



Palmitoyl CoA activates forward mode NCX1.3 activity. ***A.*** Representative macroscopic current recording of forward-mode NCX1.3 activity measured in tsA201 cells following transient transfection with NCX1.3 cDNA showing activation by high intracellular Ca^{2+} and inhibition by KB-R7943 (5 μM). ***B.*** Representative macroscopic current recording of forward-mode NCX1.3 activity in the absence (control) and presence of 1 μM palmitoyl CoA. ***C.*** Grouped data indicating that the late-to-peak current ratio is not significantly affected by KB-R7943 but is significantly increased by 1 μM palmitoyl CoA. **P*<0.05 vs. control. n = 3 patches for KB-R7943 group and 13 patches for palmitoyl CoA group. ***D.*** Grouped data showing that KB-R7943 (5 μM) significantly reduces, while palmitoyl CoA (1 μM) significantly increases total NCX1.3 forward-mode activity. **P*<0.05 vs. respective controls. n = 3 patches for KB-R7943 group and 14 patches for palmitoyl CoA group.

Activation of forward-mode NCX1.3 activity by acyl CoAs exhibits saturation and chain length dependence. ***A-E***, Representative macroscopic NCX1.1 current recordings showing that short chain (decanoyl CoA, C10:0) and polyunsaturated acyl CoAs (linoleoyl CoA, C18:2 and DHA CoA, C22:6) do not significantly modulate forward-mode NCX1.3 activity, unlike stearoyl CoA (C18:0) and oleoyl CoA (C18:1). ***F.*** Grouped data showing the maximum effect of each acyl CoA on the peak current obtained during activation. $*P<0.05$ vs. control. n = 4–14 patches per group. ***G.*** Grouped data showing the maximum effect of each acyl CoA on the inactivation process as measured by the late-to-peak current ratio. The late current was measured as the steady-state current reached following 1 minute of activation. $*P<0.05$ vs. respective control. n = 4–14 patches per group. ***H.*** Grouped data indicating that saturated and monounsaturated acyl CoAs significantly increase total NCX1.3 forward-mode activity while polyunsaturated and medium chain acyl CoAs do not. $*P<0.05$ vs. respective control patches in the absence of acyl CoA. n = 4–13 patches per group.

Figure 5-2.

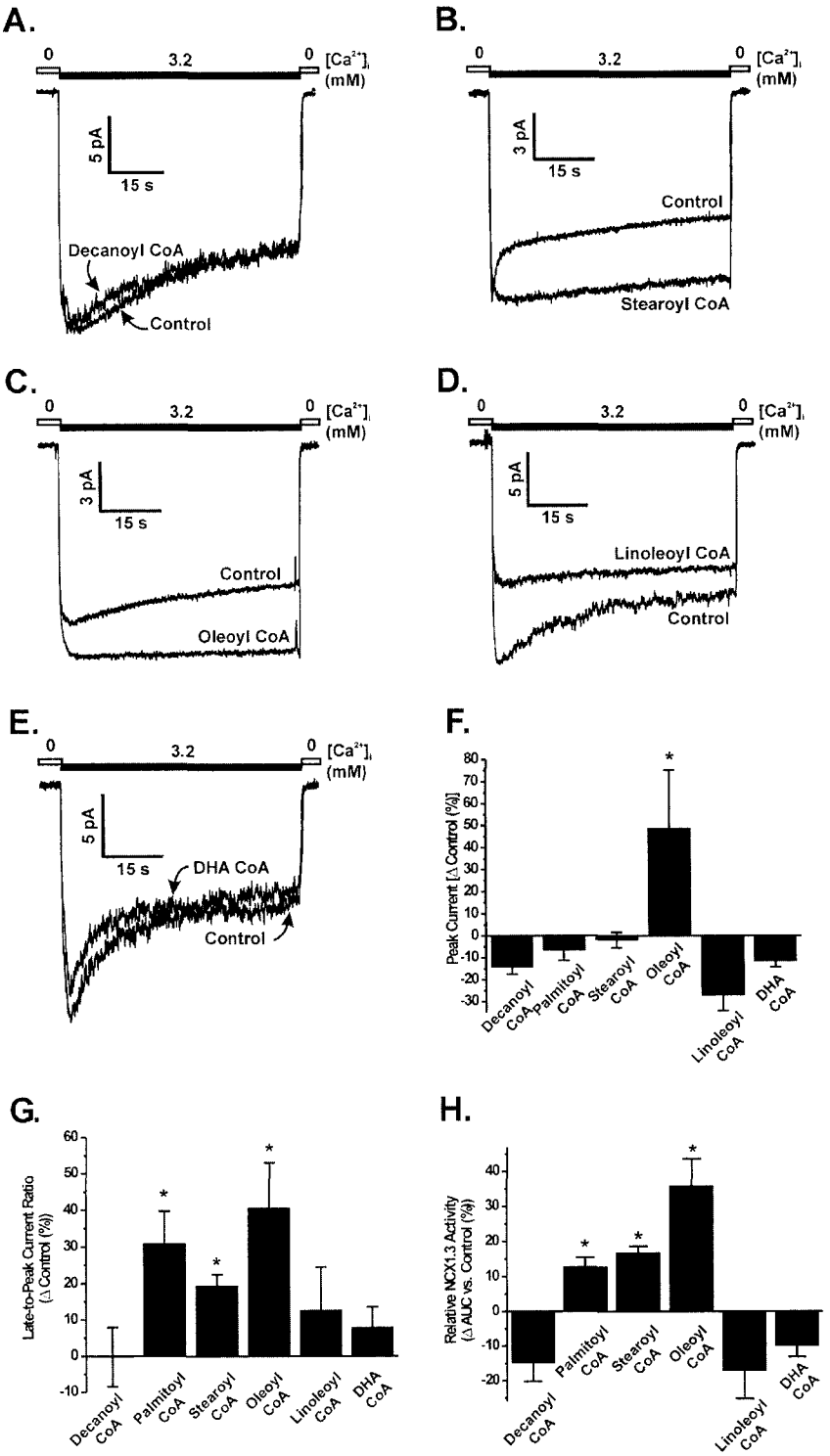
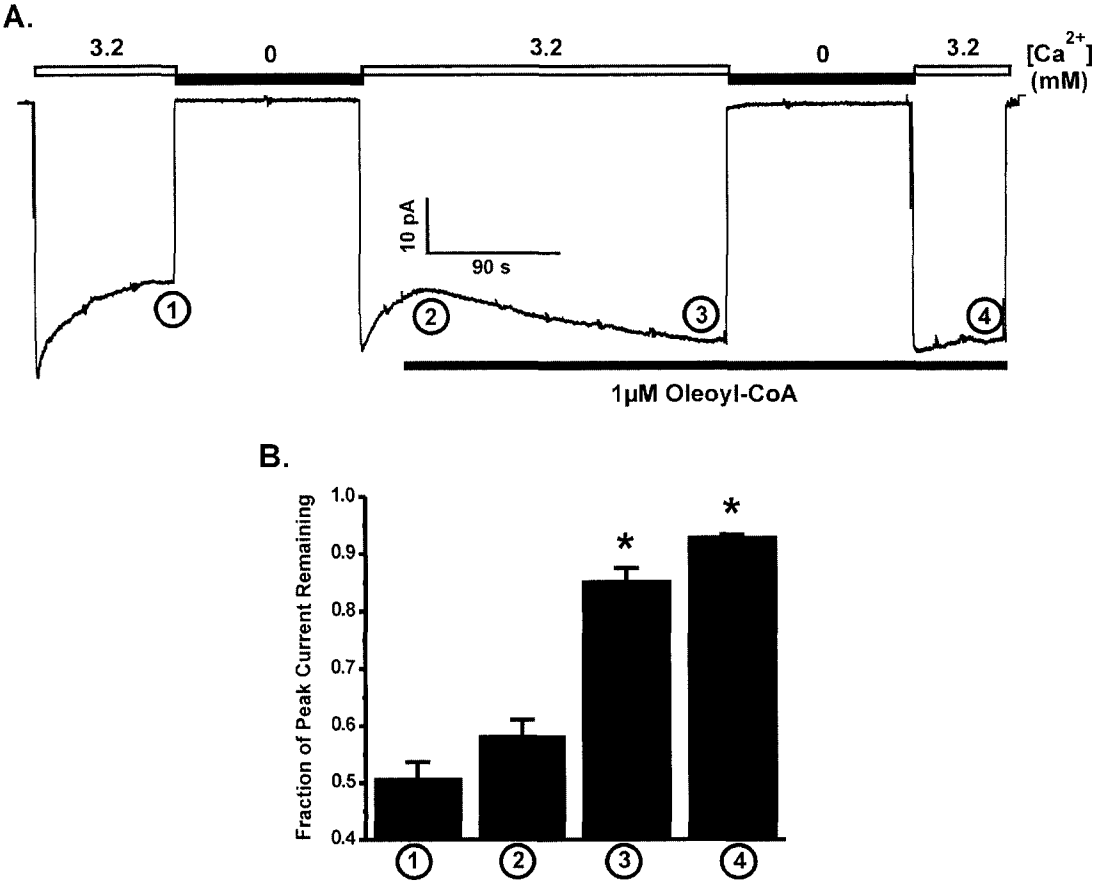


Figure 5-3.



Super-fusion of membrane patches following forward-mode NCX1.3 inactivation with oleoyl CoA reverses the inactivation process. ***A.*** Representative macroscopic forward-mode NCX1.3 current recording indicating that the inactivation process is reversible in the presence of oleoyl CoA. ***B.*** Grouped data were obtained for peak currents normalized to the peak current obtained during the control activation. Each numbered bar corresponds to the respective number on the representative trace in panel A. *$P<0.05$ vs. **circled 1** in panel ***A***. n = 3-4 patches per group.

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Chapter 6

General Discussion

Portions of this chapter have been previously published in *Human Genetics* under the following citation:

Riedel,M.J., Steckley,D.C., and Light,P.E. (2005). Current status of the E23K Kir6.2 polymorphism: Implications for type-2 diabetes. Human Genetics *116*(3):133-145.

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The various roles of fatty acid metabolites as signaling molecules have not been fully characterized. Researchers are well aware that molecules including acyl CoAs are potent modulators of ion channel/transporter function in the physiology of both health and disease. As obesity is a major contributing risk factor in the development of such pathologies as type-2 diabetes and heart disease, it is important to identify targets of lipids and lipid metabolites as well as to elucidate the underlying mechanisms that relate interaction of lipids with these targets to the development of disease.

The work presented in this thesis aims to improve our understanding of the role acyl CoAs play in the pathogenesis of type-2 diabetes and cardiac ischemia/reperfusion injury. Using a combination of molecular biology and electrophysiological techniques, this work has advanced our understanding of the molecular mechanisms for increased susceptibility to type-2 diabetes and for decreased functional recovery following periods of cardiac ischemia. More specifically, we have 1) identified a potential mechanism for the observed increase in risk for the development of type-2 diabetes in individuals harboring the homozygous E23K Kir6.2 polymorphism that involves sensitization of K_{ATP} channels to activation by long chain acyl CoAs, and 2) identified a novel mechanism for the regulation of calcium homeostasis involving acyl CoA modulation of the sodium/calcium exchanger.

It is the aim of this final discussion to provide further insight into the results obtained in an effort to relate my data to previous findings, while also attempting to foster ideas for future experiments with the goal of continuing the advancement of this line of research.

Functional consequences of the E23K Kir6.2 polymorphism

In Vivo Studies

The underlying mechanistic alterations of K_{ATP} channel function caused by the E23K polymorphism must be characterized, including the effects of heterozygous and homozygous variants, to determine the impact on development of type-2 diabetes. Studies involving human subjects have been performed by a number of groups with the common goal of demonstrating altered insulin secretion in patients with the polymorphism. The initial study by Hansen et al. showed no significant difference in first-phase insulin or C-peptide release in response to an intravenous glucose injection, nor any difference in tolbutamide-stimulated insulin secretion between individuals carrying the E23K/I337V double polymorphism and those with wild-type K_{ATP} channels (Hansen et al., 1997). However, a subsequent study revealed a slight decrease in insulin secretion during a 3 hour hyperglycemic clamp ('t Hart et al., 2002). This difference did not reach statistical significance, a problem that may have been resolved with a larger study cohort. The polygenic and multifactorial nature of type-2 diabetes and the likely small contribution to increased relative risk by any one genetic polymorphism make it difficult to detect subtle changes in glucose homeostasis in small populations. A more recent larger study suggests that insulin secretion is significantly reduced in both heterozygous (E/K) and homozygous (K/K) polymorphic individuals following an oral glucose challenge (Nielsen et al., 2003). This work has recently been confirmed by Florez and colleagues in a large scale study of 674 Scandinavian non-diabetic individuals. Siblings discordant for the E23K genotype were examined by OGTT to obtain insulinogenic index. Those siblings who were homozygous for the K allele were

found to have a 20 – 30% decrease in β-cell function when compared to siblings with an E/E or E/K genotype (Florez et al., 2004). These studies raise the possibility that the route of glucose administration (intravenous vs. oral) may determine whether the E23K polymorphism alters insulin secretion (Nielsen et al., 2003). With oral administration of glucose, additional systems that modulate the secretion of insulin and other hormones involved in glucose homeostasis are recruited, including neuronal and enteric (e.g. GLP-1) regulatory pathways (Clement et al., 2002; MacDonald et al., 2002; Miki et al., 2001; Thorens, 2003). These pathways are regulated in part by processes involving K_{ATP} channels containing the Kir6.2 subunit. It will likely be important to assess the function of these tissues in light of the potential alterations in K_{ATP} channel activity resulting from the E23K polymorphism.

Recombinant E23K Polymorphic K_{ATP} channel Studies

The functional effect of the E23K polymorphism on the properties of the K_{ATP} channel is now being investigated. In an initial study (Sakura et al., 1996), wild-type or E23K Kir6.2 subunits were co-expressed with SUR1 in Xenopus oocytes. Both the wild-type and E23K-containing K_{ATP} channels were ATP-sensitive, being similarly activated by ATP-depletion induced by metabolic inhibition. However, metabolic inhibition gives only an indication as to whether the expressed channels are sensitive to large changes in intracellular ATP levels. The limited power of this experiment does not allow for the detection of subtle yet important changes in K_{ATP} channel properties such as open probability or regulation by other K_{ATP} channel modulators that may underlie the pathophysiological relevance of the E23K polymorphism. For example, it is estimated that ~99% of K_{ATP} channels are closed in the presence of basal glucose concentrations.

Therefore, a change in K_{ATP} channel activity of less than 1% can significantly affect insulin secretion (Cook et al., 1988).

Functional studies have primarily involved the clinical assessment of insulin secretion (as measured by plasma insulin levels during a hyperglycemic clamp or oral glucose challenge) or response to sulphonylureas. E23K does not significantly affect sulphonylurea potency at the single K_{ATP} channel level or in individuals with a polymorphic genotype (Gloyn et al., 2001; Hansen et al., 1997; Schwanstecher and Schwanstecher, 2002).

It has been previously demonstrated that free nucleotide diphosphate concentrations in resting β-cells are between 10 and 20μM (Ronner et al., 2001) while resting ATP levels are in the low millimolar range (Gribble et al., 2000; Kennedy et al., 1999). E23K channels appear to have reduced ATP sensitivity in the presence of 300μM guanosine 5'-diphosphate (GDP), an activating nucleotide thought to interact only with the SUR1 subunit (Schwanstecher et al., 2002b). These results by Schwanstecher and colleagues indicate that transduction between nucleotide diphosphate binding and channel activation maybe be slightly altered in polymorphic K_{ATP} channels at low ATP and high GDP concentrations (Schwanstecher et al., 2002b), but do not support altered clinical success of sulphonylurea therapy for type-2 diabetic individuals.

Several studies have examined the structure of the K_{ATP} channel for the ATP binding site. Positively charged residues in the cytosolic N-terminus including R50 and R54 as well as the cytosolic C-terminal residue K185 are thought to interact, forming a putative ATP binding region (Cukras et al., 2002; John et al., 2003; Trapp et al., 2003; Tucker et al., 1998). Interestingly, mutations within the N-terminus encompassing amino

acids 16 – 29 were also found to significantly alter K_{ATP} channel activity (Cukras et al., 2002) suggesting that positive charged residues in this region (E23K?) may contribute to ATP binding.

Initial studies examining the effects of E23K on K_{ATP} channel function performed by Schwanstecher et al. noted an approximate 1.6-fold increase in open probability (Schwanstecher et al., 2002a). This increase in open probability in E23K polymorphic K_{ATP} channels was shown to result from a slight reduction in ATP sensitivity. The negative-to-positive change in charge that accompanies the E-to-K mutation and the proximity of E23 to residues implicated in ATP sensitivity suggests that the E23K polymorphism may alter ATP binding or the steric transduction of ATP binding to K_{ATP} channel closure.

We have performed similar studies on both wild-type and E23K/I337V double polymorphic K_{ATP} channels in the absence of ATP and obtained similar values of spontaneous open probability in both wild-type and polymorphic channels (Chapter 2 and Riedel et al., 2003). It is possible that use of the single E23K polymorphism versus the double E23K/I337V polymorphism (used in our study) could contribute to the observed differences. In addition, we were unable to reproduce the rightward shift in ATP sensitivity of polymorphic K_{ATP} channels (Schwanstecher et al., 2002a) in the absence of additional channel modulators (Riedel et al., 2003). The exact cause of this discrepancy is not currently understood, warranting further examination of the effects of nucleotides on polymorphic K_{ATP} channel behaviour.

Interestingly, it has been shown that a large shift in IC_{50} for ATP sensitivity is not a prerequisite for altered insulin secretion. Gloyn *et al.* recently described an activating

human mutation (R201H) that in the heterozygous state results in permanent neonatal diabetes mellitus (Gloyn et al., 2004). Although in the homozygous state, the mutation evoked a significant decrease in ATP sensitivity, a 1:1 mixture of mutant and wild-type channels (to simulate the heterozygous genotype) was shown to have a similar IC_{50} to that of wild-type channels. In addition, the effect on whole cell current was minimal and not significant. These results indicate that even severe mutations that result in permanent neonatal diabetes may not significantly alter IC_{50} or spontaneous open probability values. Similar to our E23K results (See chapter 2), a shift in the hill coefficient of ATP binding in the R201H mutant permitted an increase in K_{ATP} channel activity at millimolar ATP levels in a subset of the mutant K_{ATP} channel population (Gloyn et al., 2004).

The effects of acyl CoAs on E23K Polymorphic K_{ATP} channel function

The E23K variant has been linked to an increase in BMI (Nielsen et al., 2003), an indication of obesity. In individuals with elevated circulating levels of free fatty acids, including both obese and type-2 diabetic individuals, cellular accumulation of fatty acids and their metabolites, the acyl CoAs can occur (Corkey, 1988; Corkey et al., 2000; Prentki and Corkey, 1996). In β-cells, interaction of these acyl CoA molecules with the K_{ATP} channel results in increased channel activity and K^+ efflux (Branstrom et al., 1997; Branstrom et al., 1998; Gribble et al., 1998; Larsson et al., 1996). The observed increase in K_{ATP} channel current in the presence of acyl CoAs may therefore lead to a reduction in β-cell electrical excitability, contributing to the impairment of insulin secretion, a common dysfunction in type-2 diabetes.

In chapter 2, recent work is presented that examines the effects of acyl CoAs on the activity of these E23K polymorphic K_{ATP} channels. We found that the addition of

physiological (i.e. nanomolar) unbound/free concentrations (Corkey et al., 2000; Deeney et al., 1992) of palmitoyl-CoA (a common 16 carbon saturated fatty acid) resulted in a significant increase in polymorphic (E23K/I337V) K_{ATP} channel current compared to wild-type (Riedel et al., 2003). In the presence of similar acyl CoA concentrations, this would have the effect of reducing cellular excitability via increased K^+ efflux in β-cells possessing polymorphic K_{ATP} channels compared to those with wild-type channels (Figure 6-1). The increase in K_{ATP} channel activity is attributed to a reduction in the hill co-efficient of ATP inhibition, leading to a rightward shift in ATP sensitivity in the E23K/I337V polymorphic versus wild-type K_{ATP} channels at millimolar ATP concentrations (See chapter 2 and Riedel et al., 2003).

The molecular mechanism by which the E23K amino acid shift alters the ability of the K_{ATP} channel to sense changes in either ATP or acyl CoAs is currently unknown. A clear picture of the structure of the K_{ATP} channel may hold the key to determining the precise mechanism by which acyl CoAs alter channel activity and how the E23K polymorphism affects this process. The recent crystallization of related inward-rectifier potassium channels KirBac1.1 and GIRK (Kuo et al., 2003; Nishida and MacKinnon, 2002) may facilitate our understanding of the actions of polymorphisms on the binding of channel ligands such as ATP, PIP_2 and acyl CoAs, three molecules which act directly on the Kir6.2 subunit of the K_{ATP} channel (Schulze et al., 2003). While neither of these crystal structures currently includes the distal N-terminus (and therefore the E23 residue), significant progress is being made and may soon lead to a more complete model of the K_{ATP} channel at the molecular level. Indeed, our own molecular mechanistic studies suggest that the process of acyl CoA-mediated K_{ATP} channel activation involves both

binding of the negatively charged CoA head group to the protein close to the ATP binding site as well as an interaction of the long chain hydrophobic acyl tail with the plasma membrane (Manning Fox et al., 2004; Riedel and Light, 2005).

Evolutionary Aspects of the E23K Polymorphism

Given the high allelic frequency of E23K in the general population, speculation has been made regarding the evolutionary advantage of this polymorphism. Schwanstecher *et al.* first proposed that a potential discrete regulation of insulin secretion in heterozygotes as a result of slightly higher K_{ATP} channel activity may reduce glucose uptake in muscle and adipose tissue, therefore providing an evolutionary advantage by improving substrate supply for tissues with insulin-independent mechanisms of glucose uptake, especially the brain (Schwanstecher et al., 2002a). In light of recent evidence highlighting a link between increased body mass index, chronically elevated fatty acids and the E23K polymorphism (Nielsen et al., 2003), including our work on the interaction of acyl CoAs with polymorphic K_{ATP} channels (Riedel et al., 2003), we speculate further on the systemic effects of E23K and the potential advantage of maintaining this polymorphism at such high allelic frequency in the general population.

It has been previously suggested that the heterozygous E23K genotype may represent a 'thrifty gene' variant, a term coined by James Neel in 1962 (Neel, 1962). The 'thrifty gene' hypothesis suggests that during human evolution, genes primarily promoting efficient energy storage and/or utilization were preferentially selected for, resulting in improved survival during the regular periods of food shortage (Neel, 1962). In present times, the adoption of a "Western lifestyle" with abundant and easily accessible high caloric food sources and reduced exercise may predispose individuals

carrying these thrifty genes to obesity and increase their risk for developing type-2 diabetes. However, it is plausible that the homozygous wild-type (E/E) genotype may itself constitute a thrifty gene variant, given the potential for improved insulin release in response to glucose (Nielsen et al., 2003) and reduced activity of K_{ATP} channels in the absence of ATP (Schwanstecher et al., 2002a) and in the presence of acyl CoAs (Riedel et al., 2003). A reduced glucagon secretion response to hyperglycemia in E/E individuals vs. those carrying one or both E23K polymorphic alleles further supports this hypothesis (Tschritter et al., 2002). These observations suggest that carriers of the E/E genotype may be capable of more efficient energy storage, a characteristic of a 'thrifty' gene.

Recent population studies indicate there remains an unexpectedly high 11% average occurrence of this polymorphism in the glucose-tolerant Caucasian population ('t Hart et al., 2002; Gloyn et al., 2001; Gloyn et al., 2003; Hani et al., 1998; Hansen et al., 1997; Inoue et al., 1997; Nielsen et al., 2003; Sakura et al., 1996; Table 1-1). These data indicate that there may have been some evolutionary advantage to maintaining the K/K allelic combination. It has been proposed by Chakravarthy and Booth that genes promoting improved muscular performance and the efficient utilization of fuels may also be considered 'thrifty' (Chakravarthy and Booth, 2004). In accordance with this notion, we speculate that the high prevalence of the K/K genotype represents not a classical 'thrifty-storage' gene but perhaps more appropriately a 'thrifty-utility' gene, one that confers improved substrate supply for all tissues, and improved muscle performance during sustained exercise (Figure 6-2). The reasons for this suggestion are outlined as follows. Studies have shown that in K/K individuals, glucagon secretion is increased (Tschritter et al., 2002), insulin secretion is decreased (Nielsen et al., 2003) and K_{ATP}

channel activity is upregulated in the presence of acyl CoAs (Riedel et al., 2003). Physiologically, this would effectively maintain slightly higher plasma glucose concentrations, improving substrate supply for glucose-utilizing tissues such as skeletal and cardiac muscle (Figure 6-2). The slight shift in threshold of insulin secretion to higher glucose concentrations may have been counter-balanced by improved insulin sensitivity in the skeletal muscles of our ancestors, an effect that can be mimicked today by continuous endurance training (Dela et al., 1992; Russell et al., 2003). Improved insulin-independent glucose uptake via contraction-induced activation of AMP-regulated protein kinase (AMPK; Bergeron et al., 1999; Hayashi et al., 1998; Kurth-Kraczek et al., 1999) also contributes to improved substrate utilization in exercising muscle. Studies to date have failed to strongly associate the E23K polymorphism with alterations in fasting glucose or insulin levels ('t Hart et al., 2002; Nielsen et al., 2003), however there is evidence of slightly reduced fasting serum insulin levels in individuals carrying the homozygous K/K genotype (Hansen et al., 1997). A more recent study has also found a trend towards lower fasting insulin levels in a small cohort of obese children (BMI <29) harboring the homozygous K/K genotype (Le Fur et al., 2005). A significantly higher fasting glucose concentration was also found in obese children who were homozygous for both the E23K polymorphism as well as the class III VNTR alleles in the promoter region of the insulin gene (Le Fur et al., 2005). Unfortunately, a direct comparison of obese and age-matched lean children was not performed in this study. Therefore, additional large-scale studies designed to examine fasting glucose and insulin levels are warranted.

Activation of K_{ATP} channels has further been implicated in optimizing skeletal muscle contractility during exercise through increasing blood flow and potentiating force

development via increased extracellular K^+ levels (Gramolini and Renaud, 1997; Renaud, 2002). Interestingly, a recent study has shown that decreased pH results in a relative decrease in ATP sensitivity in recombinant Kir6.2(E23K)/SUR2A (skeletal muscle and cardiac type) K_{ATP} channels versus wild-type (Li et al., 2005). As anaerobic metabolism produces excess protons and decreases muscle pH, one can speculate that a population of polymorphic K_{ATP} channels may open more easily in response to an anaerobic exercise stimulus than a population of wild-type channels. Sensitization of the E23K polymorphic K_{ATP} channel to a decrease in pH may therefore improve skeletal muscle performance in these individuals. In addition, K_{ATP} channels may be activated in the heart during stress, thereby reducing action potential duration and improving cardiac function in response to β-adrenergic stimulation – improving the 'fight or flight' response (Zingman et al., 2002). It has also been suggested by Goodwin and Taegtmeyer (2000) that this response may be strengthened in the presence of elevated acyl CoAs. They have shown that the increase in acyl CoA levels that occurs during exercise results in improved β-oxidation and substrate supply required to maintain energy homeostasis in the contracting heart (Goodwin and Taegtmeyer, 2000). Taken together, these results suggest that increased activity of K_{ATP} channels, as observed in the presence of the E23K polymorphism (Riedel et al., 2003; Schwanstecher et al., 2002a), may result in improved cardiac and skeletal muscle performance during exercise. The K/K genotype may therefore have conferred an evolutionary advantage to our 'hunter-gatherer' ancestors by allowing them to maintain intense physical activity for prolonged periods of time such as was required for the procurement of food through hunting (Cordain et al., 1998). The evolutionary downside to possessing the E23K polymorphism may become apparent when homozygous

individuals adopt a modern "Western lifestyle," i.e. reduced physical activity coupled with increased fat and carbohydrate intake, increasing their likelihood of developing obesity and contributing to the onset of type-2 diabetes. The selection against the E23K polymorphism may not have occurred in our ancestral past as enforced fasting and obligatory physical activity likely balanced periods of high caloric intake and physical inactivity.

The evolutionary benefits of each homozygous genotype – the K/K 'thrifty-utility' vs. the E/E 'thrifty-storage' gene (Figure 6-2) – may be balanced in heterozygotes, contributing to the high prevalence of the E/K genotype in both the general and type-2 diabetic population (Gloyn et al., 2003; Hani et al., 1998; Nielsen et al., 2003) and in present times conferring only a slight increase in risk for the development of type-2 diabetes (Love-Gregory et al., 2003; Schwanstecher and Schwanstecher, 2002).

Physiological Relevance of the E23K Polymorphism

There is now strong evidence to suggest that the frequency of the homozygous E23K polymorphism is higher in Caucasian type-2 diabetic individuals than in the general Caucasian population (Gloyn et al., 2003; Hani et al., 1998). However, it currently remains unclear how the E23K polymorphism precisely contributes to the development of the type-2 diabetes. Although genetic predisposition is considered a major risk factor, a significant contribution also originates from environmental factors such as diet and lifestyle. For example, our recent findings that acyl CoA sensitivity is significantly increased in polymorphic K_{ATP} channels (Chapter 2 and Riedel et al., 2003) provides a plausible explanation as to why alterations in glucose homeostasis are seen

only in certain studies. These results coupled with those of Schwanstecher et al. reporting increased K_{ATP} channel activity in the presence of physiological nucleotide concentrations (Schwanstecher et al., 2002a) indicates that the E23K polymorphism may alter the binding of several channel modulators. Interestingly, a potential link has been made between the presence of E23K and increased BMI (Nielsen et al., 2003). This is in contrast to an earlier report by Hansen and colleagues in which it was stated that no interaction between obesity (defined as BMI>25) and E23K could be detected. An examination of the populations used in each of these studies yields a possible explanation for the discrepancy. In the study by Hansen and colleagues (1997), the population was comprised of 346 young adults with average BMI values in the range of 22 to 24. The low BMI values among participants may have prevented the authors from noting any effects due to obesity. Conversely, participants in the Nielsen study had BMI values above 25 and there was a noted significant difference between BMI values of individuals with wild-type K_{ATP} channels versus those that were homozygous for the E23K polymorphism (Nielsen et al., 2003). In vivo studies dedicated to examining the effects of elevated acyl CoAs due to obesity have yet to be completed.

K_{ATP} channels are involved in the secretion of multiple hormones that regulate plasma glucose levels. The expression of E23K polymorphic K_{ATP} channels in many tissues will likely affect cellular processes including glucagon and GLP-1 secretion, CNS-mediated appetite regulation, and cardiac and skeletal muscle function. Individuals homozygous for the E23K polymorphism who possess elevated acyl CoA levels may exhibit reduced responsiveness in these multiple tissues possibly contributing to chronic hyperglycemia. The finding that E23K associates with individuals with increased BMI

(Nielsen et al., 2003) supports this hypothesis. Recent studies have indicated that both insulin (Nielsen et al., 2003) and glucagon (Tschritter et al., 2002) secretion is altered in individuals carrying the E23K polymorphism. Additional studies in extra-pancreatic tissues will be required to fully understand the impact of the polymorphism on glucose homeostasis and energy balance. Furthermore, it will be important to design transgenic animal models of the homozygous E23K polymorphism allowing for the characterization of the polymorphism in multiple tissues as well as in response to such environmental stresses as dietary manipulation and exercise.

Recent studies have addressed the issue of acute versus chronic FFA exposure to otherwise healthy individuals with or without a family history of type-2 diabetes (Kashyap et al., 2003). It was found that both insulin secretion rate and plasma C-peptide levels significantly decreased in patients with a family history only after being given a multi-day lipid infusion to chronically raise plasma FFA levels to those seen in obese and type-2 diabetic individuals (Kashyap et al., 2003). Accordingly, it has been recently shown that long-term (24 hour) fatty acid infusion leads to significant reductions in first-phase insulin secretion in glucose-intolerant relatives of type-2 diabetic individuals but not in glucose-tolerant controls (Storgaard et al., 2003). The observed decrease in first-phase insulin secretion in these studies is consistent with the involvement of the K_{ATP} channel-dependent mechanism of insulin secretion in the development of type-2 diabetes (Henquin, 2000). The underlying genetic difference between those individuals with and without a family history of type-2 diabetes, including any possible involvement of the E23K polymorphism, remains to be identified.

As the effects of E23K on the overall process of glucose homeostasis are likely to be discrete, sufficiently large studies will be required to separate out differences between wild-type, heterozygous, and homozygous polymorphic individuals. Florez and associates indicate that 120,000 case/control pairs may be required to properly analyze the effects of the E23K polymorphism on type-2 diabetes susceptibility (Florez et al., 2004). Due to the multifactorial nature of type-2 diabetes, these studies may require the separation of populations based on the presence or absence of multiple risk factors, including the degree and type of obesity, gender, age, and ethnic background. For example, it will be important to examine additional populations that exhibit increased susceptibility to type-2 diabetes, including North American Indians such as the Oji-Cree (Hegele et al., 2003) and Pima (Knowler et al., 1990) populations as well as those individuals with a family history of type-2 diabetes.

Rational Experimental Design Based on Crystal Structure Models

In chapter 3, recent work examining the effects of acyl tail chain length and degree of saturation was detailed. Our lab and others have found that a critical chain length of approximately 14 – 16 Angstroms (12 – 14 carbons) is required to elicit K_{ATP} channel activation (Branstrom et al., 2004; Riedel and Light, 2005). The elucidation of crystal structures of related potassium channels has allowed for prediction of the putative tertiary structure of the Kir6.2 subunit (Figure 6-3A), which has recently been validated by electron density mapping (Mikhailov et al., 2005). This model in turn, allows us to examine in more detail the potential interactions of acyl CoA molecules with the channel itself. As eluded to in chapter 3, we believe that there is an important interaction of the acyl CoA head group with specific residues in the cytosolic portion of the Kir6.2 subunit.

These residues, including R54 and R176 may form a region of interaction for the negatively charged head group (Figure 6-3B). Appropriate positioning of the head group within this pocket may position the hydrophobic tail to then insert into the membrane and affect channel activity.

A more detailed examination of the K_{ATP} channel model reveals some interesting numbers (Figure 6-3C). Drawing a direct line from the centre of the R54/R176 cluster to the membrane yields a distance of approximately 10 Å. This approximates the distance between the phosphate groups of the CoA head group and the sulfur atom that serves as the thiol-ester linkage point for the hydrophobic acyl tail (Figure 6-3D), suggesting that an interaction of the acyl tail with the membrane is possible. An examination of the primary and tertiary structure in this vicinity reveals a potentially important cysteine residue (C166) located just inside the transmembrane domain (Figure 6-3C). The distance from the same centre of R54/R176 to this cysteine residue is slightly greater (11 – 18 Å depending on which subunit possesses the cysteine of interest). These distances are comparable to that between phosphate groups on the CoA head group and the sulfur atom to which the acyl tail is connected (Figure 6-3D) and allow us to form a working hypothesis regarding the interaction of the acyl CoA molecule with the K_{ATP} channel protein. First, the distances between the charged atoms on R54 and R176 range between 3 and 9 Å, as do the distances between the phosphate groups in the CoA head group. It is therefore possible for the different phosphate groups to interact directly with R54 and R176 in such a manner that the acyl tail is then positioned to interact optimally with the α-helices that span the membrane. Additional experimental and modeling work will be required to properly dock the acyl CoA molecule within its appropriate binding site on

the channel before we can confidently propose this mechanism. Automated docking programs that use energy minimalization to calculate ligand-protein interactions are available and can serve as powerful tools to estimate the placement of the acyl CoA molecule within its binding region. Additionally, it is conceivable that the proximity of the C166 residue with the CoA sulfur atom may facilitate an auto-acylation of this residue.

Protein acylation, commonly referred to as palmitoylation or thioesterification, is a process that transfers the fatty acid tail from an acyl CoA molecule to a cysteine-containing peptide. This process can be mediated by a variety of acyltransferases (Gutierrez and Magee, 1991; Berthiaume and Resh, 1995) or it can occur spontaneously in the absence of enzymes (Bizzozero et al., 2001). The prerequisites for auto-acylation include an elevated concentration of substrate, in this case any given acyl CoA, and a mechanism that brings the sulfur residue on the acyl CoA molecule in close proximity to the cysteine residue, which can be facilitated by the presence of positively charged amino acids (Bizzozero et al., 2001). Our suggested interaction of acyl CoAs with the K_{ATP} channel, and in particular the interaction of the CoA head group with the positively charged R54, R176 and possibly K23 residues seem to satisfy both of these criteria. It is therefore interesting to speculate that a transfer of the acyl tail to the channel itself may occur and could contribute to the activation of the K_{ATP} channel. In fact, interaction of free fatty acids to the α-helices of the KcsA potassium channel have been shown to cause altered spatial positioning that could lead to a change in channel activity (Williamson et al., 2003). Interestingly, and as shown in Figure 1-3, free fatty acids, when applied directly to the K_{ATP} channel, result in channel inhibition. The lack of the CoA head

group in the free fat precludes the possibility of an acylation event occurring under these circumstances. It is therefore possible that in the presence of the free fat, an interaction occurs at a site different than that of the hydrophobic tail of the acyl CoA.

The possibility exists that the process of auto-acylation may account for some of our observations detailed in chapter 3. It was noted that saturated and *trans* acyl CoAs led to a sustained activation of K_{ATP} channel activity while activation by a similar length cis monounsaturated acyl CoA was readily reversible. As noted in chapter 3 this may result from a differential mobility of these acyl CoAs in the membrane and may lead to higher local concentrations at area of increased membrane curvature such as surrounds the K_{ATP} channel (Cohen et al., 2003). We speculate that this increased local concentration of saturated and *trans* acyl CoAs may better position them to partake in an auto-acylation event. Transfer of the acyl tail to the channel may then lead to a more sustained irreversible activation response. During the washout period, there is no substrate provided (free CoA) that could participate in the reverse reaction, thus the fatty acid tail may remain bound to the channel indefinitely. This mechanism is currently purely speculative and experiments are being designed to test our hypothesis. This includes the creation of a point mutation at residue 166, converting the wild-type cysteine residue to a valine. Valine lacks the sulfur atom required for acylation to occur and yet does not significantly alter K_{ATP} channel ATP sensitivity or gating kinetics (Trapp et al., 1998). This yields a powerful tool for studying the involvement of acylation at C166 and may provide us with the first experimental evidence of acylation of the K_{ATP} channel. Additional examination of wild-type and C166V mutant K_{ATP} channel acylation using

fluorescently labeled palmitoyl CoA could provide additional evidence for this mechanism. These experiments remain to be performed in the near future.

Interestingly, several cysteine residues in the cytosolic portion of NCX have also been identified. These cysteine residues appear to be important in regulating NCX activity (Ren et al., 2001) and may be involved in governing the effect of acyl CoAs on this membrane protein. NCX activity is altered by agents such as methanethiosulfonate (MTS) that prevent cysteine reactivity. We speculate that palmitoylation of the exchanger at one or more of these cysteine residues could regulate basal function and that use of these agents could disrupt acyl CoA binding thus reducing NCX activity. Much more work is required to validate this hypothesis, including the much anticipated elucidation of the crystal structure of the NCX1 protein.

Implication of This Work for Other Tissues

Intracellular acyl CoA levels are dynamically regulated and therefore serve as important signaling molecules in a number of physiological processes. The identification of novel ion transport targets of acyl CoA modulation is important for the progression of our understanding of lipid-mediated regulation of cellular ionic homeostasis. In chapters 4 and 5, we have identified the sodium/calcium exchanger as a novel target of acyl CoA regulation. We present detailed information regarding the potential role of acyl CoA-mediated modulation of NCX activity in both the pancreatic β-cell and in the cardiomyocyte. The majority of the work presented was performed in recombinant systems, investigating the interaction of acyl CoAs with NCX at the molecular level. While it was beyond the scope of my project, these studies provide us with rationale to expand our findings into a more physiological context (i.e. primary tissues) in which acyl

CoAs and NCX are simultaneously present. In these additional tissues, we can speculate further on physiological relevance.

One particular tissue of interest is the brain. As a result of mechanisms similar to that in cardiac tissue, brain ischemia can lead to activation of reverse-mode NCX activity via neuronal proton and subsequent sodium loading. Neuronal damage sustained as a result can lead to the observed motor and memory deficits that accompany ischemic brain injury. Interestingly, it has been shown that specific activation (using $FeCl_3$) and inhibition (using XIP) of NCX activity in a model of brain ischemia can exert a protective or detrimental effect, respectively (Pignataro et al., 2004). The proposed mechanism for protection of neurons by NCX involves the activation of forward-mode operation in surrounding neurons that had not been infarcted. In these neurons, continued extrusion of calcium was thought to prevent calcium overload-induced injury.

During a cerebral ischemic episode, free fatty acid levels are dramatically elevated (Rabin et al., 1997). This, as in other tissues, would likely lead to a cytosolic accumulation of acyl CoAs as detailed elsewhere in this thesis (see chapter 1). We have shown that in the presence of acyl CoAs, forward-mode NCX1.1 activity is inhibited while reverse-mode NCX1.1 activity is enhanced. Acyl CoA-mediated activation of reverse-mode NCX1.1 may therefore provide an additional mechanism for the observed neuronal damage in the ischemic region. Conversely, we have shown that acyl CoAs increase NCX1.3 forward-mode activity (see chapter 5), indicating that acyl CoA modulation of NCX is isoform specific. Currently however, NCX1.3 has not been identified in the brain (Quednau et al., 1997), necessitating the study of acyl CoA effects on additional NCX1 isoforms.

The K_{ATP} channel may also play a role in preventing neuronal damage during cerebral ischemic injury. Evidence exists that over-expression of Kir6.2, and subsequent over-activity of the K_{ATP} channel in the forebrain can protect against ischemic brain injury (Heron-Milhavet et al., 2004). In this case, the mechanism of protection may involve a hyperpolarization of neurons in the infracted area. Studies have shown that by resting or hyperpolarizing neurons that are deprived of oxygen, functional recovery can be improved (Centonze et al., 2001). The hyperpolarizing influence of increased K_{ATP} channel activity could contribute to this protective mechanism. Again, in the event of brain ischemia, free fatty acid levels are increased and may lead to a cytosolic accumulation of acyl CoAs (Rabin et al., 1997). The subsequent activation of K_{ATP} channels by elevated acyl CoAs could further enhance this hyperpolarizing influence. Therefore, in the brain, activation of K_{ATP} channels by acyl CoAs may be an inherent protective mechanism, while in the pancreatic β-cell, this same mechanism may contribute to the development of type-2 diabetes.

As mentioned above, GLP-1 secretion from intestinal L-cells is also governed in part by K_{ATP} channel activity (Reimann and Gribble, 2002; Gribble et al., 2003). The activation of K_{ATP} channels by acyl CoAs could limit the secretion and effectiveness of this hormone in the following manner. First, as in the β-cell, the mechanism for GLP-1 secretion involves a metabolic inhibition of K_{ATP} channel activity followed by membrane depolarization (Reimann and Gribble, 2002; Gribble et al., 2003). Therefore, as in the β-cell, over-activity of K_{ATP} channels in the presence of elevated acyl CoAs could potentially reduce the secretion of the hormone. Secondly, a major mechanism of action of GLP-1 is activation of the cAMP/PKA pathway and phosphorylation-mediated

inhibition of K_{ATP} channels (MacDonald et al., 2002). In the event of elevated acyl CoAs, the level of K_{ATP} channel inhibition required to sufficiently depolarize the membrane to its threshold potential may be increased. In this case, more GLP-1 may be required to exert its effect on the β-cell or conversely, higher glucose levels may be required to elicit an insulin secretion response. This mechanism of reduced GLP-1 effectiveness under conditions of elevated acyl CoAs has yet to be tested and may provide an addition trigger for reduced insulin secretion in obese individuals with type-2 diabetes. This mechanism may be overcome with the administration of GLP-1 receptor agonists and intensive research is being conducted to bring this type of treatment to the clinic (Holst, 2004).

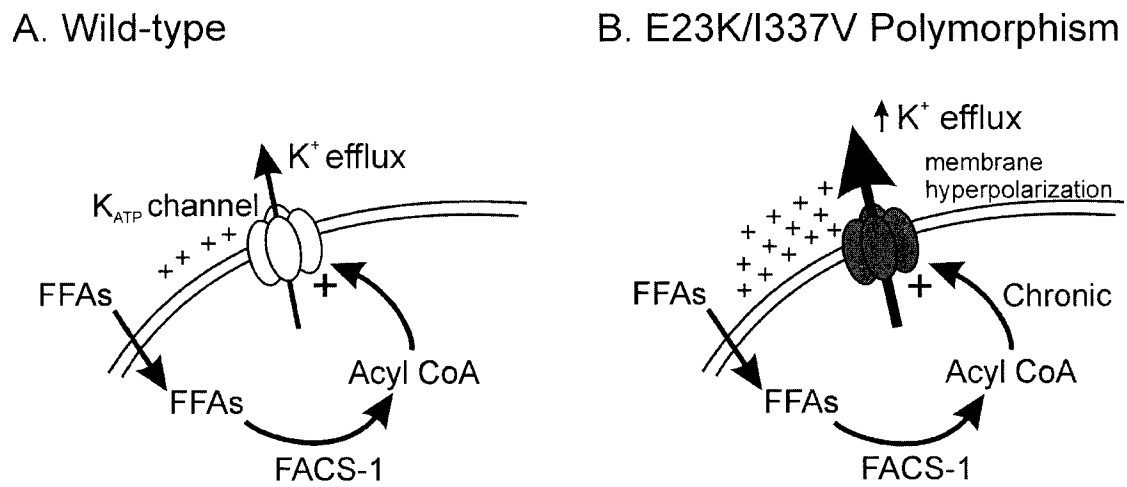
Summary and Conclusions

Much work remains to be completed before we can truly understand the implications of elevated acyl CoAs on the development of type-2 diabetes. While the recombinant experimental data presented in this thesis points to an important role of saturated and trans acyl CoAs in mediating β-cell dysfunction and reduced functional recovery in post-ischemic cardiac tissue, validation of this work will be required in more intact systems. The isolation of primary islets and β-cells and the use of the FACS-1 virus to augment intracellular acyl CoA levels in an intact cell system will be a powerful tool to confirm the results presented here. Functional studies using intact isolated cardiomyocytes exposed to the FACS-1 virus may yield an additional model in which we can test the effects of elevated acyl CoAs on ischemic damage in the cardiac setting. These technologies are readily available and will hopefully form the basis of many projects within the lab in the near future. The creation of transgenic animals expressing

the E23K Kir6.2 polymorphism will provide further tools to assess the role of this mutation in a number of conditions including following high fat diet feeding and exercise regimes. Using these models, the role of the E23K polymorphism could be examined in multiple tissues and pharmacological treatments could be tested for effectiveness. It will be interesting to see these projects develop.

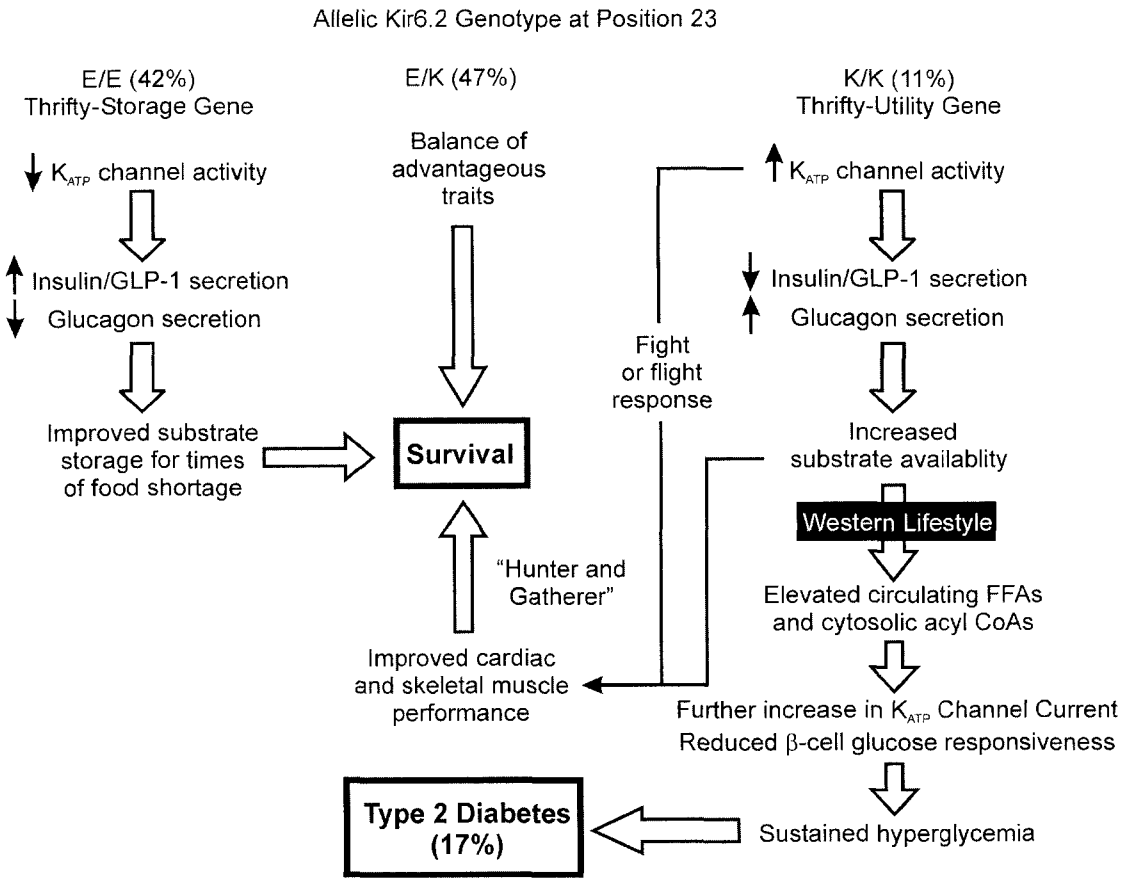
In conclusion, this thesis has identified the sodium/calcium exchanger as a novel target of lipid modulation and has advanced our understanding of the molecular mechanisms governing the interaction of acyl CoAs with a previously identified target, the β-cell K_{ATP} channel. The work presented here has direct implications for the dietary management of individuals at high risk for ischemic heart disease and type-2 diabetes and suggests that screening individuals for the E23K polymorphism may identify addition at risk populations. The implications for screening for the E23K polymorphism are evident when one considers the burden of cost on the health care systems of developed nations struggling with their waistlines and the epidemic of type-2 diabetes. Prevention is the ultimate cure for type-2 diabetes.

Figure 6-1.



Long chain acyl CoA esters such as palmitoyl-CoA directly activate K_{ATP} channels. Chronic exposure to free fatty acids results in cytosolic accumulation of acyl CoAs and increased K_{ATP} channel activity, a situation that may lead to impaired insulin secretion. K_{ATP} channels carrying the homozygous K/K genotype are more susceptible to activation by acyl CoAs. An increased K^+ efflux may reduce β-cell activity, contributing to hyperglycemia and type-2 diabetes.

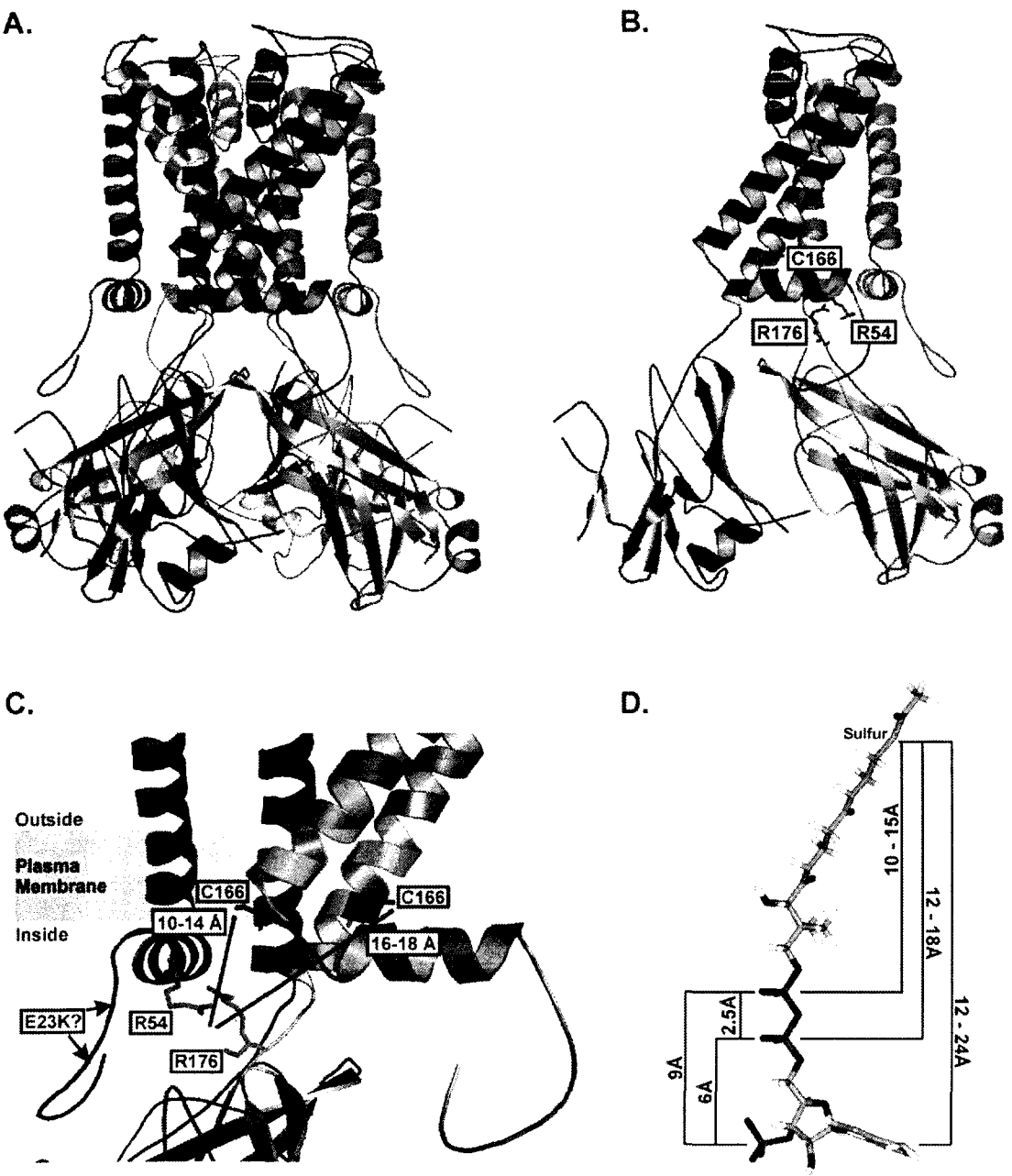
Figure 6-2.



Schematic representation of the selection pressure that maintains a high prevalence of the various genotypes at residue position 23 of the Kir6.2 subunit of the K_{ATP} channel. Given in parentheses are the average genotypic frequencies of each allelic combination in the general Caucasian population as calculated in Table 1. The evolutionary advantage of the K/K genotype may be lost in the presence of a 'Western Lifestyle.' A high caloric diet coupled with reduced physical activity leads to increased risk for type-2 diabetes which in turn may contribute to the increased prevalence of the K/K genotype within this population. See text for full discussion.

Molecular models of Kir6.2 subunit tertiary structure. ***A.*** Proposed tertiary structure of the Kir6.2 tetramer based on the crystal structure of the related Kirbac1.1 bacterial potassium channel. ***B.*** Two adjacent subunits are shown with three potentially important residues of the binding of acyl CoAs labeled. ***C.*** A closer look at the putative acyl CoA binding region. Residues R54 and R176 are thought to be involved in binding of the CoA head group while C166 may participate in an auto-acylation event, linking the fatty acyl tail to the channel protein. Distances were measured using the PyMol measurement wizard and are represented graphically here. The ranges correspond to the measurements taken from different charged atoms on the starting residue. The potential location of the E23K Kir6.2 polymorphism is also indicated, although the current models do not contain structural information for the proximal N-terminus. ***D.*** A graphic representation of the acyl CoA head group. Distances between phosphate groups and the sulfur atom are indicated. The ranges indicate that bond angles are adjustable and the exact folding of the molecule *in vivo* is unknown.

Figure 6-3.



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