From the Rolf Luft Center for Diabetes Research
The Endocrine and Diabetes Unit
Department of Molecular Medicine
Karolinska Institutet, Stockholm, Sweden

Metabolic signals and the ATP-sensitive potassium channel in the pancreatic ß-cell

By

Robert Bränström, M.D.

Stockholm 1999

Printed by REPRO PRINT AB, Stockholm, Sweden
ISBN 91-628-3479-7

ABSTRACT

ATP-sensitive K^+ (K_{ATP}) channels are widely distributed in a variety of tissues and cell types. K_{ATP} channels are comprised of two subunits, a pore forming subunit (Kir6.x) and a regulatory sulfonylurea receptor (SURx). Both subunits are required for fully functional channels. In the present thesis the role of the pancreatic ß-cell K_{ATP} channel, with regard to oscillations in electrical activity and modulation of channel activity by various compounds as well as the functional organization of channel subunits, were investigated. In the ß-cell the K_{ATP} channel (comprised of Kir6.2 and SUR1) couples metabolic changes to electrical activity. Although electrophysiological studies during the last decade have provided clues to the complex control of the K_{ATP} channel by various nucleotides and pharmacological agents, the metabolic signals responsible for its physiological regulation remain to be clarified. The widely accepted model of glucose-induced insulin secretion involves a number of events. Following glucose metabolism, the ATP/ADP ratio increases, inducing closure of the K_{ATP} channel, depolarization of the ß-cell plasma membrane and thereby opening of voltage-gated Ca^{2+} channels. The rise in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) triggers insulin release. Oscillations in some or all of these signals are thought to play an important role in the pulsatile nature of insulin release. The molecular mechanism underlying these oscillations is unknown but is an important issue since absence of normal oscillations in plasma insulin levels is observed in non-insulin dependent diabetes mellitus. In this thesis, it is shown that K_{ATP} channel activity is oscillating in intact ß-cells under stimulatory concentrations of glucose, thereby inducing fluctuations in electrical activity and $[Ca^{2+}]_i$. This effect is likely to reflect oscillations in metabolism, since blocking of glucose metabolism induced openings of the K_{ATP} channel that correlated with periods of hyperpolarization and lowering in $[Ca^{2+}]_i$. Glucose is metabolized through glycolysis in the cytosol and through the tricarboxylic acid cycle in the mitochondria. The relative role of mitochondrial metabolism has frequently been studied using a-ketoisocaproate as substrate. In contrast to previous findings, assuming that a-ketoisocaproate needs to be metabolized in order to close the K_{ATP} channel, it is shown that a-ketoisocaproate reversibly and in a dose-dependent manner inhibits the K_{ATP} channel directly. Besides glucose, free fatty acids (FFA) have been demonstrated to play a critical role in insulin secretion. It is shown that the metabolic active form of FFA, long-chain CoA (LC-CoA) esters, are accumulated intracellularly in response to prolonged exposure to elevated FFA, and are potent and chain-length specific activators of the K_{ATP} channel. Using a truncated version of Kir6.2 (Kir6.2? C26), which generates channels in the absence of SUR1, it was shown that LC-CoA esters interact directly with Kir6.2. These findings verify that LC-CoA esters have a unique binding site on the K_{ATP} channel complex and may be of physiological importance. Finally, K_{ATP} channels are organized as heterooctameric complexes assembled with a 4:4 stoichiometry of Kir6.2 and SUR1. It is shown that SUR1 plays a critical role in the functional organization of Kir6.2 by a mechanism distinct from the ability of SUR1 to recruit Kir6.2 to the membrane and the ability to convey sensitivity to sulfonylurea and MgADP.

Key Words: ATP-regulated K^+ channel, pancreatic ß-cell, oscillations, a-ketoisocaproate, long chain acyl-CoA esters, Kir6.2/SUR1 structure-function.

ISBN 91-628-3479-7

CONTENTS

LIST OF ARTICLES

This thesis is based on the following articles, and will be referred to in the text by their roman numerals:

I. Larsson, O., Kindmark, H., Bränström, R., Fredholm, B. and Berggren, P.-O. Oscillations in K_{ATP} channel activity promote oscillations in cytoplasmic free Ca^{2+} concentration in the pancreatic ß cell.
Proc. Natl. Acad. Sci. U.S.A. 1996; **93**, 5161-5165.

II. Bränström, R., Efendic, S., Berggren, P.-O. and Larsson, O. Direct Inhibition of the Pancreatic ß-Cell ATP-regulated Potassium Channel by a-Ketoisocaproate.
J. Biol. Chem. 1998; **273**, 14113-14118.

III. Larsson, O., Deeney, J.T., Bränström, R., Berggren, P.-O. and Corkey, B.E. Activation of the ATP-sensitive K^+ Channel by Long Chain Acyl-CoA: A role in modulation of pancreatic ß-cell glucose sensitivity.
J. Biol. Chem. 1996; **271**, 10623-10626.

IV. Bränström, R., Corkey, B.E., Berggren, P.-O. and Larsson, O. Evidence for a Unique Long Chain Acyl-CoA Ester Binding Site on the ATP-regulated Potassium Channel in Mouse Pancreatic Beta Cells.
J. Biol. Chem. 1997; **272**, 17390-17394.

V. Bränström, R., Leibiger, I., Leibiger, B., Corkey, B.E., Berggren, P.-O. and Larsson, O. Long Chain Coenzyme A Esters Activate the Pore-forming Subunit (Kir6.2) of the ATP-regulated Potassium Channel.
J. Biol. Chem. 1998; **273**, 31395-31400.

VI. Bränström, R., Leibiger, B., Moede, T., Lindqvist, P., Leibiger, I., Berggren, P.-O. and Larsson, O. The Sulfonylurea Receptor Promotes Functional Orientation of the K_{ATP} Channel Pore Forming Subunit Kir6.2.
Submitted.

Some additional data, not previously published have been included in the results and discussion chapter.

ABBREVIATIONS

ACBP	Acyl-CoA binding protein
AMP, ADP and ATP	Adenosine mono-, di-, and tri-phosphate
BCH	ß-2-aminobicyclo[2.2.1]heptane-2-carboxylic acid
BIM	Bisindolylmaleimide
$[Ca^{2+}]_i$	Cytoplasmic free calcium concentration
CoA	Coenzyme A
IDDM	Insulin dependent diabetes mellitus
EC_{50}	Concentration causing half-maximal effect
FABP	Fatty acid binding protein
FFA	Free fatty acid
GFP	Enhanced green-fluorescent protein
IC	Current-clamp
K_{ATP}	ATP-sensitive K^+ channel
K_{BK}	Big conductance, voltage and Ca^{2+}-activated K^+ channel
KCO	K^+ channel openers
a-ketoisocaproate	4-methyl-2-oxopentanoate
Kir	Inwardly rectifying K^+ channel
K_{SK}	Small conductance K^+ channel
LC-CoA ester	Long-chain acyl CoA ester
NADH	Nicotinamide adenine dinucleotide
NaN_3	Na-Azide
NBF	Nucleotide binding fold
NIDDM	Non-insulin dependent diabetes mellitus
PIP_2	Phosphatidylinositol 4,5-*bis*-phosphate
PKC	Protein kinase C
P_O	Open probability
VC	Voltage-clamp
V_m	Membrane potential
S	Siemens (Conductance)
SUR	Sulfonylurea receptor

INTRODUCTION

All living cells are surrounded by a lipid membrane which separates the world within the cell from its exterior. In the membrane there are ion channels which constitute fast and efficient signalling systems. These channels consist of mono- or multimeric protein complexes and allow passage of charged atoms like Na^+, K^+, Ca^{2+} and Cl^-. The flow of ions through a single ion channel induces very small currents, in the low picoampere range (10^{-12} A). Ion channels have been shown to have great influence on cell functions under normal physiological and pathophysiological conditions. With the development of the patch-clamp technique in the early 1970's by Sakmann and Neher, it became possible to study these channels in more detail. Several ion channels are regulated by receptors, localized at the actual ion channel complex, which upon activation affects opening and closing of the channel. One example of this is the ATP-sensitive K^+ (K_{ATP}) channel which is the main focus of this thesis.

K_{ATP} channels are widely distributed in a variety of tissues and cell types. They were first discovered in cardiac myocytes (Noma 1983) and later found in the pancreatic ß-cell (Cook and Hales, 1984), smooth (Standen *et al.*, 1989) and skeletal muscle (Spruce *et al.*, 1985), brain (Ashford *et al.*, 1988), pituitary (Bernardi *et al.*, 1993) and kidney (Hunter and Giebisch, 1988). K_{ATP} channels have also been identified in mitochondria (Inoue *et al.*, 1991). The K_{ATP} channel acts as a link between the metabolic state of the cell and electrical activity thereby regulating a variety of cellular functions. In the pancreatic ß-cell, where the functional role of K_{ATP} channels is best understood, the channel couples glucose metabolism to insulin secretion (Ashcroft and Rorsman, 1990). In smooth muscle cells excitability has been shown to be affected by the activity of K_{ATP} channels (Quayle and Standen, 1994). In cardiac myocytes and neurons, activation of K_{ATP} channels have been shown to be involved in cytoprotection following ischemia (Gross and Auchampach, 1992; Grover *et al.*, 1992), whereas its role under normal physiological conditions remains controversial. It is clear that the regulation of K_{ATP} channels is of central importance in many cell systems under both physiological and pathophysiological conditions.

Structure and regulation of the K_{ATP} channel family - Cloning studies have demonstrated that K_{ATP} channels are heteromultimers of two different subunits; a large subunit of 145 kD that binds sulfonylurea (SURx) (Aguilar-Bryan *et al.*, 1995) and a small subunit of 36 kD which belongs to the inwardly rectifying K^+ (Kir) channel family (Kir6.x) (Inagaki *et al.*, 1995a, b). Reconstitution experiments have demonstrated that both

subunits are required to form active K_{ATP} channels (Inagaki *et al.*, 1995a). SURx belongs to the ATP-binding cassette (ABC) superfamily which also includes the multidrug resistance-associated protein (MDR) and cystic fibrosis transmembrane conductance regulator (CFTR). SURx has two nucleotide binding folds (NBFs), both with high sequence similarity to other members of the ABC-superfamily. Both NBFs harbor the Walker A and B consensus motifs, involved in nucleotide binding (Walker *et al.*, 1982). Two isoforms of SURx have been identified, SUR1 and SUR2 (Aguilar-Bryan *et al.*, 1995; Kurachi *et al.*, 1996). The SUR2 gene can generate at least two splice variants, designated SUR2A and SUR2B (Chutkow *et al.*, 1996). SUR1 exhibits high affinity binding for sulfonylureas and is found in neuroendocrine cells like pancreatic ß-cells as well as in certain regions of the brain (Aguilar-Bryan *et al.*, 1995). The two low-affinity receptors SUR2A and SUR2B are thought to constitute the K_{ATP} channels in cardiac, skeletal and smooth muscle cells (Fig. 1). An exact membrane topology of SURx has not yet been determined experimentally. Computer-predicted topology, using either hydropathy analysis or multi-sequence alignment with other members of ABC-superfamily proteins, have suggested a 13 or 17 transmembrane spanning domain organization, respectively (Aguilar-Bryan *et al.*, 1995; Tusnady *et al.*, 1997). In addition, mutations directed towards the nucleotide binding folds (NBFs) decrease the sensitivity for cytosolic nucleotides (Gribble *et al.*, 1997), suggesting that the two NBFs are localized intracellularly (Fig. 2*B left*). In this context, it should be mentioned that mutations in the NBFs have been shown to cause persistent hyperinsulinemic hypoglycemia of infancy (PHHI) (Aguilar-Bryan *et al.*, 1995).

An inwardly rectifying K^+ channel subunit (Kir), that forms the pore of K_{ATP} channels, was first cloned using a fragment of Kir3.4 as cDNA probe (Inagaki *et al.*, 1995b). This new Kir channel, named Kir6.1, had a 40-50% amino acid sequence similarity with other previously cloned members of the Kir-superfamily and represented a new subfamily, Kir6.x. Kir6.1 is present predominantly in the inner membrane of mitochondria but is not expressed in the insulin-secreting cell lines HIT-T15, RINm5F and MIN6 (Suzuki *et al.*, 1997b), all of which have plasma membrane K_{ATP} channels. Therefore it was not surprising, using Kir6.1 cDNA as probe, that a novel Kir subunit (currently referred to as Kir6.2) was cloned (Inagaki et al., 1995a). Kir6.2 has ~70% amino acid sequence similarity with Kir6.1. Kir6.2 is expressed at high levels in pancreatic islets and insulin producing cell lines (Suzuki *et al.*, 1997a). Co-expression of Kir6.2 with SUR1 results in a channel with identical functional and pharmacological properties to the native pancreatic ß-cell K_{ATP} channel (Inagaki *et al.*, 1995a). Channels reconstituted from

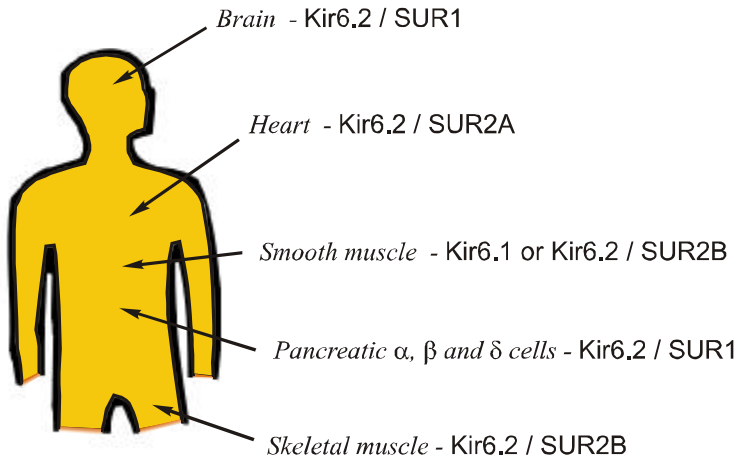

Figure 1. ATP-sensitive K^+ channels are found in a variety of tissues. The various Kir and SUR subunits interact to form K_{ATP} channels with different pharmacological and nucleotide sensitivity.

Kir6.2 and SUR2A resemble the cardiac- and skeletal muscle cell type K_{ATP} channel (Inagaki *et al.*, 1996) whereas Kir6.x/SUR2B has been proposed to consitute the channels found in smooth muscle cells (Fig. 1). The human SUR1 gene is localized to the short arm of chromosome 11 (11p15.1) with the gene encoding Kir6.2 at the 3´ end (Inagaki *et al.*, 1995a) and indicates that they have been fused into one protein earlier in evolution (Aguilar-Bryan *et al.*, 1998). The human SUR2 gene has been localized to chromosome 12 (12p12.1) (Chutkow *et al.*, 1996). Sequence analysis of the Kir6.x family has predicted two transmembrane spanning domains, M1 and M2, flanking the K^+ pore loop (P-loop) (Fig. 2 and 13). The Kir channels, as their name implies, are inwardly rectifying channels that transduce K^+ ions better in the inward than outward direction (see Fig. 2*A left*). Under normal ionic gradients K^+ current flow outward at membrane potentials positive to the K^+ equilibrium potential. The single channel conductance, measured in the inside-out patch at symmetrical K^+ concentrations, ranges between 50-80 pS (see review by Ashcroft and Ashcroft, 1990) for inward currents and the single-channel current-voltage (*i-V*) relation is linear. For outward currents, the *i-V* relation shows a pronounced rectification and the current increases little at membrane potentials more positive than +20 mV. It has been shown that rectification primarily results from a voltage-dependent block of outward currents by internal cations, mainly Na^+ and Mg^{2+} (Findlay, 1987a). In the P-loop of Kir6.x a highly conserved H5 region motif is found, as in all inwardly rectifying K^+ channels identified to date, that contributes to the ion selectivity and rectification properties

(Polonsky *et al.*, 1988). Point mutation close to the P-loop (N160D) converts the weakly rectified Kir6.2 to exhibit strong rectification (Shyng *et al.*, 1997a).

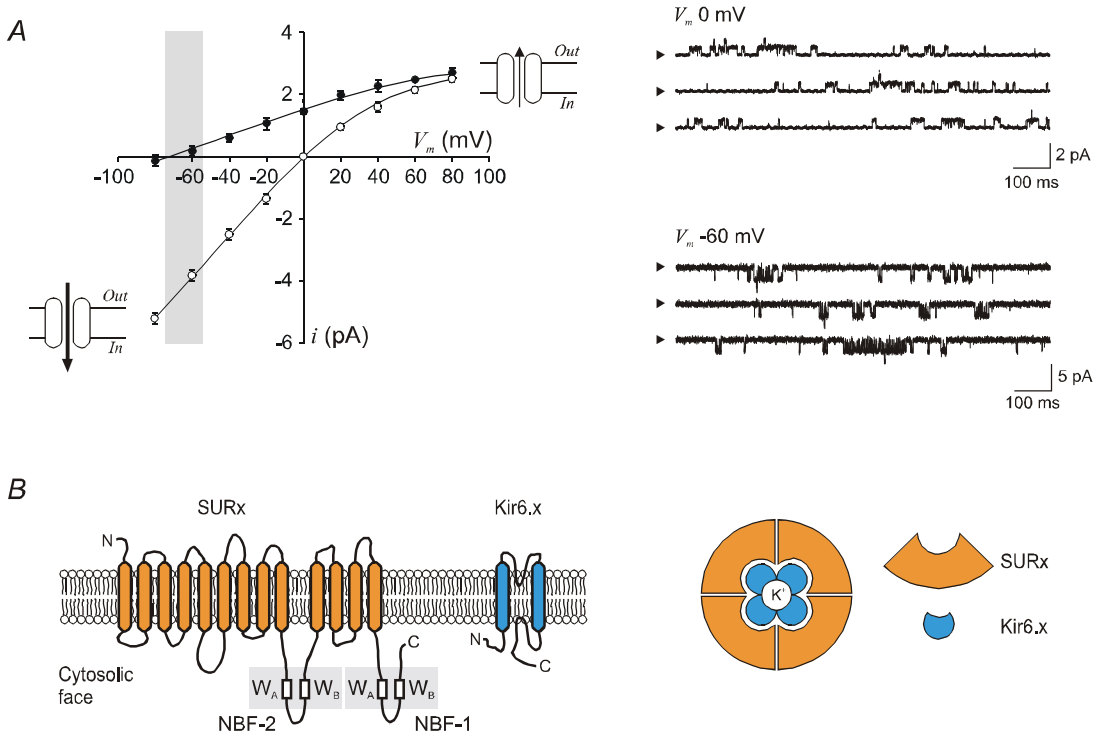

Figure 2. Characteristics of the K_{ATP} channel. ***A***, single K_{ATP} channel current-voltage relations (*left*) and kinetics (*right*) measured in an inside-out patch isolated from a pancreatic ß-cell. At normal ionic gradients ($[K^+]_i$. 150 mM, $[K^+]_o$. 5 mM; filled circles) single-channel conductance is -20 pS and no bursts are seen (*top*). Using symmetrical K^+ gradients ($[K^+]_i$. 150 mM, $[K^+]_o$. 150 mM; open circles) single-channel conductance is -70 pS and channel openings are grouped in bursts (*bottom*). As indicated, the channel transduces K^+ ions better in the inward than outward direction. Greyed area indicates the resting membrane potential of the pancreatic ß-cell. ***B***, predicted membrane topology and stoichiometry of SURx and Kir6.x subunits. Transmembrane domains are represented by cylinders. *Left*, using hydropathy analysis it has been suggested that SURx has 13 membrane spanning domains. Two nucleotide binding folds (NBF-1 and NBF -2) are indicated on the cytosolic part of SURx. Walker A (W_A) and Walker B (W_B) motifs are denoted within the NBFs. Kir6.x has N- and C-termini facing the intracellular space. *Right*, highly schematized model of the heterooctameric stoichiometry of the K_{ATP} channel. Four Kir6.x and four SURx form one functional K_{ATP} channel complex.

Members of the Kir superfamily have been proposed to assemble into tetrameric complexes (Glowarzki *et al.*, 1995; Yang *et al.*, 1995), i.e. four Kir subunits assemble to form one K^+ conductive pore. Using fusion constructs of wild-type and modified Kir6.2, which exhibit weak and strong rectification, fused and unfused with SURx, it was shown that Kir6.2 and SUR1 assemble as a tetrameric complex with a 4:4 ratio to form one K_{ATP} channel complex (Fig. 2*B right*; Shyng and Nichols, 1997c; Clement *et al.*, 1997). Thus,

K_{ATP} channels are the first example of a heteromultimeric complex assembled by an ion channel and a receptor that are structurally unrelated to each other.

Even though almost 15-years have past since the discovery of K_{ATP} channels, their physiological regulation is still not fully understood. As the name implies, K_{ATP} channels are characterized by an inhibition of channel activity when the ATP concentration at the cytoplasmic surface is increased (Noma, 1983; Cook and Hales, 1984; Rorsman and Trube, 1985). Electrophysiological studies have shown that the kinetic and pharmacological properties of K_{ATP} channels, such as single channel conductance, ATP sensitivity and responses to sulfonylureas and K^+ channel openers (KCO), vary among different tissues (Ashcroft, 1988; Quast and Cook, 1989; Davis *et al.*, 1991). The reported half-maximal inhibitory concentration (EC_{50}) for ATP on Kir6.2/SUR1 is between 5 and 15 µM (Cook and Hales, 1984; Ashcroft *et al.*, 1987b; Inagaki *et al.*, 1995a), whereas for cardiac myocytes the K_{ATP} channel (Kir6.2/SUR2A) it is between 20-200 µM (Noma, 1983; Nichols and Lederer, 1991; Terzic *et al.*, 1995). A number of studies have demonstrated that phosphorylation is not involved in channel inhibition by ATP, since non-hydrolyzable analogues such as AMP-PNP, AMP-PCP and ATP?S are effective inhibitors and ATP inhibits in the absence of Mg^{2+} (Cook and Hales, 1984; Larsson *et al.*, 1993). In inside-out patches (see *Electrophysiology*) K_{ATP} channel activity decreases rapidly following patch excision (*run-down*). Addition of ATP in the presence of Mg^{2+} can prevent or reverse *run-down*, whereas ATP^{4-} and non-hydrolyzable analogues are not able to refresh channel activity. Thus, phosphorylation is probably required to maintain channel activity, but not channel inhibition. Recent data have suggested that the K_{ATP} channel is not phosphorylated itself, but instead that ATP is involved in phosphorylating PI to PIP and PIP_2, which in turn may serve as K_{ATP} channel activators (Hilgemann and Ball, 1996; Shyng and Nichols, 1998; Baukrowitz *et al.*, 1998; Xie *et al.*, 1999).

Agents capable of modulating ATP-sensitivity are likely to be of importance in regulating channel activity, since the K_{ATP} channel can be expected to be completely closed at physiological, millimolar concentrations of ATP (Kakei et al., 1986; Vogt *et al.*, 1998). Several nucleotides have been reported to increase K_{ATP} channel activity in the presence of ATP. These include ADP, UDP and GDP. Kir6.2/SUR1 channels are less sensitive than those comprised of Kir6.x/SUR2 for nucleotide diphosphates such as UDP and GDP (Seino, 1999), and it has been proposed that UDP and GDP play an essential role in regulating K_{ATP} channel activity in tissues containing SUR2. In the pancreatic ß-cell, most studies have concentrated on the action of ADP since the concentrations of ATP and ADP

change reciprocally in response to glucose metabolism (Ashcroft and Rorsman, 1990; Erecinska *et al.*, 1992; Matschinsky, 1996). Nucleotides that are able to stimulate channel activity only do so provided Mg^{2+} is present in the millimolar range (Dunne and Petersen, 1986a, b; Findlay, 1987b).

Both NAD/NADH and NADP/NADPH induce channel activation at low concentrations and channel inhibition at high concentrations (Dunne *et al.*, 1988). The effects of pyridine nucleotides are complex and their physiological significance remains unclear. Besides direct regulation of K_{ATP} channel activity by adenine, guanosine and pyridine nucleotides, it has been shown that several other signal transduction pathways may be involved in channel regulation. Protein kinase C (PKC), in particular, has been suggested to be involved, whereby activation of PKC leads to K_{ATP} channel activation in cardiac myocytes (Hu *et al.*, 1999) or inhibition in pancreatic ß-cells (Wollheim *et al.*, 1988). However, no obvious correlation between different K_{ATP} channel isoforms and PKC activation has so far been established. One explanation could be the fact that endogenous PKCs exist as a broad range of isoforms with different tissue distributions, Ca^{2+} and substrate specificity (Shearman *et al.*, 1989).

Since the cloning of the K_{ATP} channel subunits, it has been possible to dissect which subunits are involved in regulating channel inhibition and activation. It has thereby been proposed that the primary ATP-inhibitory site lies on the Kir6.2 subunit and that the SURx subunit increases the sensitivity to ATP and endows MgADP and MgGDP sensitivity (Tucker *et al.*, 1997; Trapp *et al.*, 1997). Several point mutations in Kir6.2 have been shown to decrease the sensitivity to ATP several-fold, but it is still debatable whether Kir6.2 harbors the functional ATP-binding site, since expression of Kir6.2 alone leads to an EC_{50} for ATP that corresponds to ~100 µM, and the fact that co-expression with SUR1 increases ATP-sensitivity approximately 10-fold. Disruption of the Walker A or B motifs in SURx dramatically decreases channel activation by MgADP and MgGDP, suggesting that the NBFs in SURx are involved in dinucleotide-induced activation of K_{ATP} channels (Aguilar-Bryan *et al.*, 1995; Nichols *et al.*, 1996; Gribble *et al.*, 1997; Shyng *et al.*, 1997b). However, analysis following mutagenesis requires careful interpretation because a point mutation might alter the nucleotide sensitivity in several ways; altering Kir6.x and SURx interactions, interfering with the transduction mechanism within the channel complex by which ligands induce channel openings and closings and finally, it could affect the binding site itself.

Several drugs are known to potently affect K_{ATP} channel activity. Best known are sulfonylurea compounds, used for almost four decades in the treatment of non-insulin dependent diabetes mellitus (NIDDM). Sulfonylureas fall into two major groups, the first generation drugs of which tolbutamide is the best studied and a second generation of drugs such as glibenclamide and glipizide. All sulfonylurea compounds are able to block channel activity even when applied to the outside of the cell and it has therefore been proposed that they reach their target by dissolving in the lipid face of the plasma membrane (Trube *et al.*, 1986). The potency of sulfonylureas increases with their lipophilicity, further supporting this notion (Gylfe *et al.*, 1984). For many years the mechanism of action of these compounds remained unclear. With the identification of K_{ATP} channels in the mid 1980's, it was demonstrated that sulfonylureas potently and specifically inhibit the K_{ATP} channel (Sturgess *et al.*, 1985) and likely exerted their anti-diabetogenic effect through this action. ß-cell K_{ATP} channels (Kir6.2/SUR1) are potently inhibited by sulfonylureas - EC_{50} for glibenclamide is less than 10 nM. Binding experiments and electrophysiological studies show that K_{ATP} channels comprised of SUR2A and SUR2B are about ~50-fold less sensitive to sulfonylureas (Inagaki *et al.*, 1996; Gribble *et al.*, 1998). Besides sulfonylureas, many other substances have been identified that block K_{ATP} channel currents, examples of which are imidazoline (Zaitsev *et al.*, 1996), mexiletine (Tricarico *et al.*, 1998) and quinine (Bokvist *et al.*, 1990) with different affinity depending on the type of K_{ATP} channel studied. In addition to blockers, several pharmacological K_{ATP} channel activators (KCO) have been described. KCO comprise a structurally diverse group of drugs with a broad spectrum of potential therapeutic applications in conditions like hypoglycemia, hypertension, anti-arrhythmias, angina pectoris and asthma (see review by Lawson, 1996). KCO include diazoxide, nicorandil, minoxidil, pinacidil and cromokalim. Of these, diazoxide is perhaps the most important, since it has been used clinically in the treatment of insulin producing tumors, PHHI and hypertension (Goode *et al.*, 1986). The Kir6.2/SUR1 and Kir6.2/SUR2B channels are both activated by diazoxide whereas the Kir6.2/SUR2A channels are not (Inagaki *et al.*, 1996). Instead, K_{ATP} channels in cardiac and smooth muscle (SUR2A and SUR2B) are potently activated by pinacidil and cromakalim (Weston, 1989), whereas K_{ATP} channels composed of SUR1 is poorly responsive to these compounds. The exact locations of sulfonylurea and diazoxide binding motifs on SURx have not yet been established, but these compounds are likely to bind to distinct sites (Bray and Quast, 1992). Diazoxide has been proposed to interact at the NBFs since point mutations to these regions severely decrease the ability to activate the channel (Aguilar-Bryan *et al.*, 1995; Nichols *et al.*, 1996; Gribble *et al.*, 1997).

The kinetics of K_{ATP} channels are complex and vary between different channel isoforms and experimental condition. With normal ionic gradients ($[K^+]_o$. 5 mM and $[K^+]_i$. 155 mM) current flow is outward at membrane potentials more positive than . -85 mV (the K^+-equilibrium potential). Under these conditions channel kinetics are characterized by openings which can be described by two open states and one closed state (Fig. 2*A right*, Bokvist *et al.*, 1990; Larsson *et al.*, 1993) (see statement 4, *Materials and Methods*). However, the majority of studies on K_{ATP} channel kinetics have been performed on inward currents at negative membrane potentials (between -60 and -100 mV) using high extracellular K^+ concentration. Under these conditions, channel openings are grouped in bursts (Fig. 2*A right*). The lifetime of the bursts and of the closed intervals which separate bursts are quite variable between different types of K_{ATP} channels. In contrast, channel open and closed times within bursts as well as single channel conductance for Kir6.2/SUR2A are similar to those of Kir6.2/SUR1 (Inagaki *et al.*, 1996), indicating that these properties are determined by Kir6.2. Furthermore, it has been suggested that SURx determines the burst duration and interburst interval, since these kinetic parameters are much longer for Kir6.2/SUR2A channels than those of Kir6.2/SUR1 channels (Inagaki *et al.*, 1995a; Alekseev *et al.*, 1997).

The K_{ATP} channel in the pancreatic ß-cell - Insulin is exclusively produced by the pancreatic ß-cell, located within the islets of Langerhans. Insulin is the only hormone capable of lowering blood glucose levels and lack of insulin or relative insensitivity to insulin in target organs will result in diabetes mellitus. There are two main types of diabetes mellitus; insulin dependent diabetes mellitus (IDDM), an autoimmune disease leading to a total lack of insulin producing ß-cells and an absolute requirement of daily injections with insulin, and non-insulin dependent diabetes mellitus (NIDDM), a disease with impaired insulin secretion as well as peripheral insulin resistance. About 90% of all diabetics are suffering from NIDDM, a disease associated with high morbidity in affluent societies. NIDDM is often paralleled with increased tissue triglycerides and/or elevated circulating levels of free fatty acids (Lewis *et al.*, 1972; Fraze *et al.*, 1985).

The pancreatic ß-cell is unique in that nutrients, like glucose, have to be metabolized in order to stimulate insulin secretion. A model of the stimulus-secretion coupling in the ß-cell has been developed over the last three decades and is schematically presented in figure 4. In this model, glucose enters through the glucose transporter (GLUT-2) (Meglasson and Matschinsky, 1986) and is phosphorylated by glucokinase, the first enzyme in the glycolytic pathway which also serves as a glucose sensor with a K_m for

glucose of 10 mM (Meglasson *et al.*, 1986). Following glucose metabolism by glycolytic enzymes in the cytosol and the tricarboxylic acid cycle (Krebs cycle) in the mitochondria, an increase in ATP/ADP ratio (Kakei *et al.*, 1986; Malaisse and Sener, 1987) and reduced pyridine nucleotides (Pralong *et al.*, 1990) are seen. The activity of K_{ATP} channels in the ß-cell is thought to be regulated by the ratio between ATP and ADP, where ATP inhibits (Rorsman and Trube, 1985; Arkhammar *et al.*, 1987; Ashcroft and Rorsman, 1989) and ADP activates (Kakei *et al.*, 1986; Dunne and Petersen, 1988; Larsson *et al.*, 1993), and thus an increased ATP/ADP ratio closes the K_{ATP} channel. When the channels close, the K^+ flux through the membrane is reduced and subsequently the ß-cell depolarizes. Since the extracellular Ca^{2+} concentration is more than 10,000 times higher than the intracellular Ca^{2+} concentration, Ca^{2+} will enter the cell passively along its

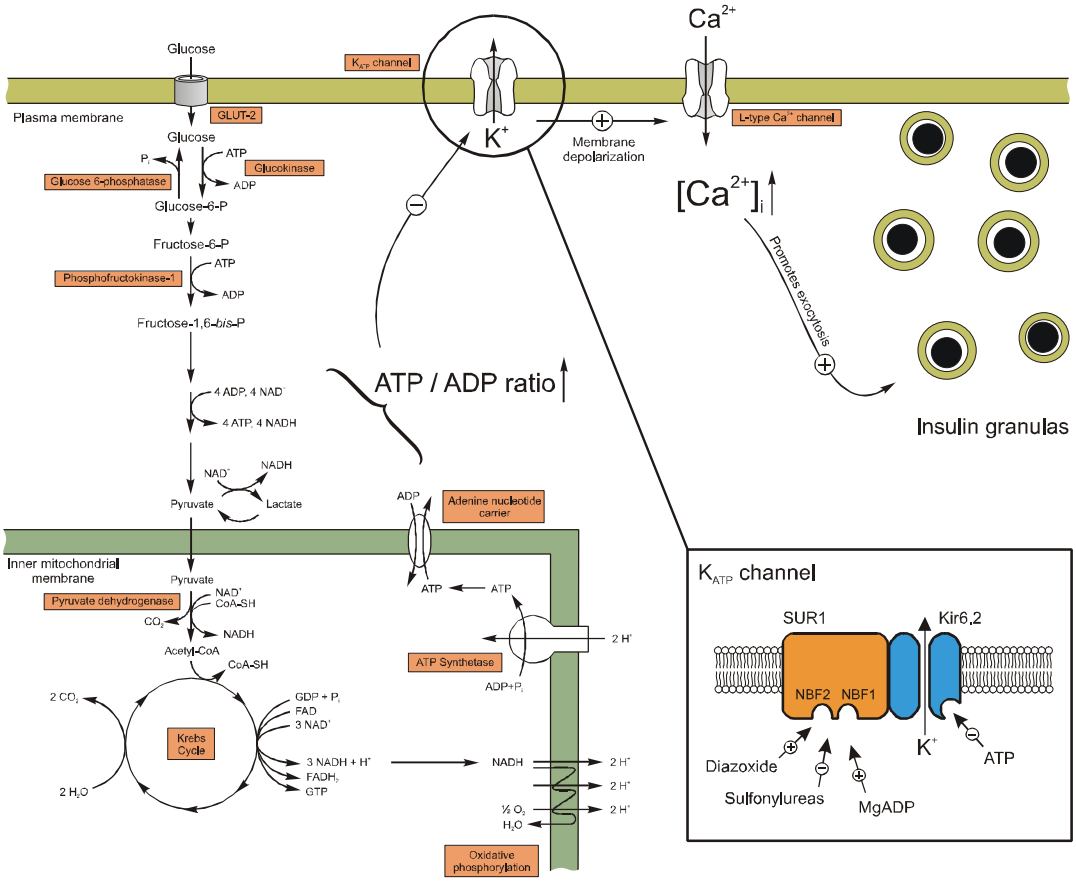

Figure 3. Schematic model of the stimulus-secretion coupling in the pancreatic ß-cell. Glucose enters via the GLUT-2 transporter. Following glucose metabolism in glycolysis and the tricarboxylic acid cycle (Krebs cycle), the ATP/ADP ratio increases, thereby blocking K_{ATP} channel activity. Closure of K_{ATP} channels will depolarize the membrane potential from .-70 mV to .-40 mV which will open voltage-gated Ca^{2+} channels and allow a rapid increase in $[Ca^{2+}]_i$ which finally triggers insulin secretion. *Inset*, current opinion on the regulation of K_{ATP} channels in pancreatic ß-cells.

electrochemical gradient. The increase in $[Ca^{2+}]_i$ will trigger insulin release. As the membrane potential reaches . -40 mV, voltage-gated Ca^{2+} channels open, which causes a train of Ca^{2+}-dependent action potentials (see Fig. 3 and 4). As in most excitable cells, several Ca^{2+} channel subtypes co-exist (Zhang *et al.*, 1993). In pancreatic ß-cells, the L-type Ca^{2+} channel is the most prominent and electrical activity as well as insulin secretion is inhibited following block of these channels (Ashcroft and Rorsman, 1990; Berggren and Larsson, 1994). ß-cells are relatively small cells with a diameter between 10-15 µm and a high input impedance, and the activity of the K_{ATP} channels determines the resting membrane potential. At low glucose concentrations membrane potential is between -70 and -60 mV (Fig. 4). The total number of open K_{ATP} channels is predicted to be 1-25% under these conditions (Ashcroft and Rorsman, 1989; Cook et al. 1988). It has been proposed that in order to depolarize the ß-cell membrane, a complete block of K_{ATP} channels is required (Arkhammar *et al.*, 1987; Cook *et al.*, 1988).

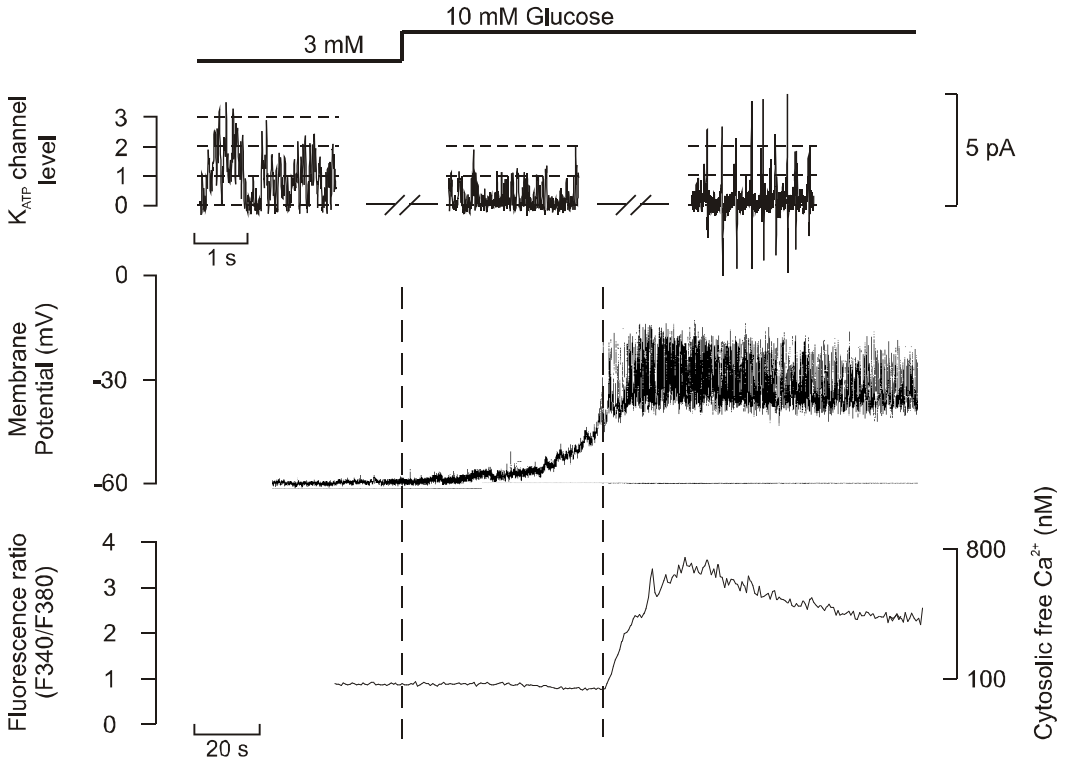

Figure 4. Recordings of K_{ATP} channel activity (*top*), membrane potential (*middle*) and cytosolic free Ca^{2+} concentration (*bottom*) from a pancreatic ß-cell (separate recordings). Increasing the glucose concentration from 3 mM (non-stimulatory) to 10 mM (stimulatory) causes the ß-cell to close the K_{ATP} channels, depolarize and exhibit action potentials. Opening of Ca^{2+} channels rapidly increase the $[Ca^{2+}]_i$ (Own unpublished observations).

Numerous studies have pointed to several levels of oscillatory behavior in the ß-cell stimulus-secretion coupling. Employing classical electrophysiological methods, Dean and Matthews (1968) were the first to record electrical activity in pancreatic ß-cells. They showed that the electrical activity in the presence of intermediate glucose concentrations (8-12 mM) has a characteristic pattern of slow oscillations in membrane potential on which bursts of action potentials are superimposed. An oscillatory pattern in insulin secretion has been described in man and animals *in vivo* (Goodner *et al.*, 1977; Matthews *et al.*, 1983), in perfused pancreas and perifused islets *in vitro* (Stagner *et al.*, 1980; Chou and Ipp, 1990). Oscillatory insulin secretion from single ß-cells has been demonstrated using amperometric techniques (Smith *et al.*, 1995; Fig. 7 in this thesis). Both $[Ca^{2+}]_i$ and mitochondrial respiration, measured as O_2 consumption, oscillate in synchrony with electrical activity and insulin release (Gilon *et al.*, 1993; Longo *et al.*, 1991; Santos *et al.*, 1991). Taken together, these observations indicate that the source of the oscillatory behavior lies within the ß-cell itself. The molecular mechanism underlying oscillations in insulin release is unknown but is an important issue since absence of normal oscillations in plasma insulin levels is observed in NIDDM (Lang *et al.*, 1981; Polonsky *et al.*, 1988).

Besides glucose, only a few physiological substrates initiate insulin release. In this context a-ketoisocaproate, the deamination product of L-leucine, is of special interest. Glucose and a-ketoisocaproate are both decarboxylated in the mitochondria, cause an increase in ATP/ADP, NAD/NADH and NADP/NADPH ratios, closure of K_{ATP} channels, depolarization and insulin release (Panten *et al.*, 1972; Hutton et al., 1980; Ashcroft *et al.*, 1987a). These similarities led to the conclusion that a-ketoisocaproate and glucose initiate insulin release via a common metabolic signal. Since a-ketoisocaproate is exclusively metabolized in the mitochondria, a signal generated from mitochondria was thought to be of crucial importance (Panten *et al.*, 1981). The consensus molecule has been proposed to be mitochondrially-generated ATP (Hutton *et al.*, 1980). Because of similarities with glucose regarding effects on catabolic activities and insulin secretion, a-ketoisocaproate has been used extensively over the last decades to study signal-transduction in the ß-cell.

The vast majority of studies on the ß-cell has focused on carbohydrate metabolism. However, a growing concept is evolving that an important signal for insulin secretion may reside in the complex interplay between glucose and lipid metabolism. Interestingly, long term exposure to elevated levels of FFA decreases glucose-induced insulin secretion from pancreatic islets and cells (Sako and Grill, 1990; Zhou and Grill, 1994), pointing to the importance of lipid metabolism in the stimulus-secretion coupling in the pancreatic ß-cell. In addition, *in vivo* studies in man have shown that glucose responsiveness is severely

decreased following 24-h infusion of non-esterified fatty acids (Paolisso *et al.*, 1995). One possible explanation could be that FFA, or metabolic products derived from lipid metabolism, modulate the K_{ATP} channel and thereby insulin secretion. Long-chain CoA acyl (LC-CoA) esters have been suggested as a signal molecule involved, since there is a good correlation between LC-CoA levels and decreases in insulin secretion (Prentki *et al.*, 1992). LC-CoA esters increase following increased levels of extracellular FFA (Fraze *et al.*, 1985) or following release from intracellular lipid stores (Malaisse *et al.*, 1983). Glucose has been shown to decrease cellular content of LC-CoA esters acutly (Prentki *et al.*, 1992). However, it has been proposed that cytosolic levels of LC-CoA esters increase following glucose stimulation via formation of malonyl-CoA which inhibits carnitine palmitoyltransferase 1 (CPT-1) (Prentki and Corkey, 1996), responsible for transporting LC-CoA esters into the mitochondria for ß-oxidation (Fig. 5). LC-CoA esters and products from them have been shown to be potent regulators of enzymes and channels (Table 1).

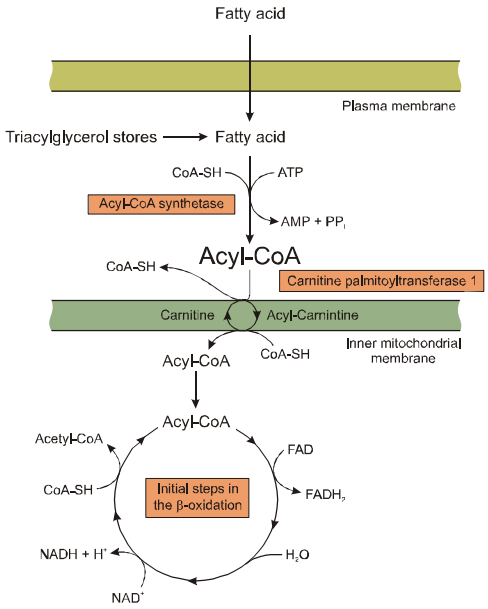

Figure 5. Key-steps in fatty acid activation, transport across the mitochondrial inner membrane and ß-oxidation.

Table 1. LC-CoA esters are proposed to have many functions in cell function. Examples of both stimulatory and inhibitory effects of LC-CoA esters (for details see review by Faegeman and Knudsen 1997).

Long chain acyl-CoA regulation of:	Effect:
Gene expression	
E. Coli transcription factor FadR	Inhibitory
Carnitine Palmitoyltransferase-1	Stimulatory
Ion channels / pumps	
Ca^{2+} ATPase	Stimulatory
Na^{+}/K^{+}-ATPase	Stimulatory
Protein sorting	
Vesicular transport in Golgi apparatus	Stimulatory
GTP-dependent vesicle fusion in ER	Inhibitory
Enzymes	
Acetyl-CoA carboxylase	Inhibitory
Carnitine palmitoyltransferase-1	Stimulatory
Adenine nucleotide translocase	Inhibitory
Glucokinase	Inhibitory
Pyruvate dehydrogenase	Inhibitory
Long-chain acyl-CoA dehydrogenase	Inhibitory
Hormone-sensitive lipase	Inhibitory

OBJECTIVES

1. To examine if the origin of the oscillations in electrical activity and $[Ca^{2+}]_i$ in single pancreatic ß-cells is caused by oscillations in the activity of K_{ATP} channels, and thus, possibly by oscillations in glucose metabolism.

2. To clarify the mechanism whereby the mitochondrial substrate a-ketoisocaproate affect K_{ATP} channel activity.

3. To investigate if a metabolically active form of FFA, long-chain CoA acyl esters (LC-CoA), have direct effects on K_{ATP} channel activity, and if so, identify the site(s) of action for such an effect on the K_{ATP} channel complex.

4. To investigate the role of SURx in the functional organization of the K_{ATP} channel.

MATERIALS AND METHODS

ob/ob mouse - Adult obese mice (gene symbol *ob/ob*) (Hellman, 1965) of both sexes were obtained from a local colony. Pancreatic islets from these mice contain more than 90% ß-cells (Nilsson *et al.*, 1987), containing K_{ATP} channels with normal characteristics (Arkhammar *et al.*, 1987). Before decapitation the mice were starved for 24-hours. Pancreatic islets were isolated by a collagenase technique (Lacy and Kostianovsky, 1967) and a cell suspension was prepared and washed essentially as previously described (Lernmark, 1974). The cells were resuspended in RPMI 1640 culture medium (Flow Laboratories, Scotland, UK) containing 11 mM glucose, 10% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin and 60 µg/ml gentamycin. The cell suspension was seeded into Petri dishes (Corning Glass, Corning, NY, USA) and incubated at 37EC in 5% CO_2 for 1-3 days.

Xenopus oocytes - The use of *Xenopus Leavis* oocytes as an expression system for ion channels was first described by Miledi and co-workers (1983). Since then, the *Xenopus* oocyte expression system has become a standard technique for investigations of ion channels. *Xenopus* oocytes are especially suitable for electrophysiology recordings, since it has only a few endogenous channels (the major one being a Ca^{2+} activated Cl^- channel), and faithfully expresses foreign RNA that has been injected. The methods used in paper V and VI to express the different subunits of the K_{ATP} channel are essentially the same as described by Stühmer and Parekh (1995). In short, female *Xenopus leavis* frogs were obtained from Blades Inc.,UK and kept in a container filled with regular tap water at 19EC. The animals were anaesthetized with 3-aminobenzoic acid ethyl ester (2 g/l water). One ovary was removed via minilaparotomy. The incision was sutured and the frog was allowed to recover. The ovary was mechanically separated and incubated at room temperature for 1-2 hours with 1 mg/ml collagenase type A (Boehringer Mannheim, Germany) in order to remove connective tissue and the outer cell coat, i.e. the follicular layer. Collagenase was dissolved in OR-2 buffer containing (in mM): 82.5 NaCl, 2 KCl, 1 $MgCl_2$ and 5 HEPES (pH 7.5). Oocytes stage V-VI were injected, using an Eppendorf transjector (Eppendorf, Hamburg, Germany), with 0.5-5 ng of mRNA, dissolved in 50 nl of RNase-free water, encoding mouse and hamster subunits of the K_{ATP} channel, as indicated. Oocytes were rinsed and incubated in Barth´s medium containing (in mM): 88 NaCl, 1 KCl, 1.68 $MgCl_2$, 0.47 $Ca(NO_3)_2$, 2.4 $NaHCO_3$, 0.41 $CaCl_2$ and 10 HEPES (pH 7.4) supplemented with 0.55 mg/ml Na-pyruvate, 100 IU/ml penicillin and 100 µg/ml

streptomycin. Antibiotics were purchased from Sigma. Oocytes were maintained in tissue culture at 19EC for 1-4 days. Prior to experiment the vitelline membrane of the oocyte was manually removed using fine forceps to allow access to the oocyte membrane.

Electrophysiology - The patch-clamp technique is an electrophysiological method that allows the recording of macroscopic whole-cell and microscopic single-channel currents flowing across biological membranes through ion channels. In order to record small ionic currents in the picoampere range, a low-noise recording technique is required. This was first developed by Neher and Sakmann in 1976 to investigate various ion channels in the cell membrane of giant frog muscle fibres. In 1981, the patch-clamp technique was further refined to enable high current resolution, i.e. recordings of currents from one single ion channel (Hamill *et al.*, 1981). Besides measurements of ion-channel currents, the patch-clamp technique also allows one to experimentally control and manipulate the voltage of membrane patches and whole-cells (voltage clamp or VC). Alternatively, one may monitor changes in membrane potential in response to currents flowing through ion channels (current clamp or IC). The resistance between pipette and plasma membrane is critical to decrease electrical background noise. To separate single-channel currents from background noise, the seal resistance should typically be in excess of 10^9 O (gigaohmseal or tightseal). The contact between pipette and cell membrane is mechanically very stable and allows a number of procedures leading to different recording configurations. The “cell-attached” mode is the configuration which is reached once a tightseal is reached. Withdrawing the pipette from the cell at this state, isolates the patch from its environment and exposes the cytosolic side of the membrane to the bath solution (“inside-out”). To record whole-cell currents, one most gain access to the intracellular environment. Two major possibilities are at hand, mechanical suction through the patch pipette (“whole-cell”) or by the use of pore-forming antibiotics like nystatin (Horn and Marty, 1988) or amphotericin B (Rae *et al.*, 1991), so called “perforated whole-cell”. Electrical access is obtained by pore incorporated into the patch membrane, thus providing a low resistance electrical pathway to the cell interior. The perforated whole-cell technique aims at retaining the cytosolic constituents in the cell. In the normal whole-cell configuration these constituents are diluted. A special case of patch-clamp recording is the “outside-out” configuration. This configuration allows one to easily change the extracellular side of the patch, and still record single-channel currents. This is very useful studying receptor-operated ion channels. Outside-out recordings are more difficult to obtain because more manoeuvres are required. Examples of patch-clamp configurations are shown in figure 6.

In this thesis, channel activity and membrane potential were recorded using all configurations of the patch-clamp technique. In order to reduce the background noise, the pipettes were coated with the heat sensitive polymer Sylgard resin (Dow Corning, Kanagawa, Japan) near their tips, and finally the tips of the glass pipette were fire-polished. Currents were recorded using an Axopatch 200 patch-clamp amplifier (Axon Instruments, Inc., Foster City, CA, USA). Experiments were stored on magnetic tape using a video cassette recorder (JVC, Tokyo, Japan) and a digital data recorder (VR-10B, Instrutech Corp., Elmont, NY, USA). The recorded signal was stored with an upper cut-off frequency of 2 kHz. Solutions used for inside-out patches were; standard intracellular solution (IC; i.e. bath solution) containing (in mM): 125 KCl, 1 $MgCl_2$, 10 EGTA, 30 KOH, 5 HEPES (pH 7.15), and extracellular solution (i.e. pipette solution): 138 NaCl, 5.6 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$ and 5 HEPES (pH 7.40). Single channel activity for outward currents was monitored at 0 mV. For inward currents, studied at V_m -60 mV and symmetrical K^+, the pipette solution was replaced with a modified IC containing (in mM): 140 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 5 HEPES (pH 7.40). For outside-out patches the solutions were reversed. Channel records were displayed according to the convention; upward deflections denote outward currents and *vice versa*. One hour prior to recording of membrane potential, the RPMI 1640 culture medium was replaced with regular extracellular solution containing 3 mM glucose. All experiments were performed at room temperature (20-24°C), except for membrane potential recordings, where the temperature has been varied as indicated in the text.

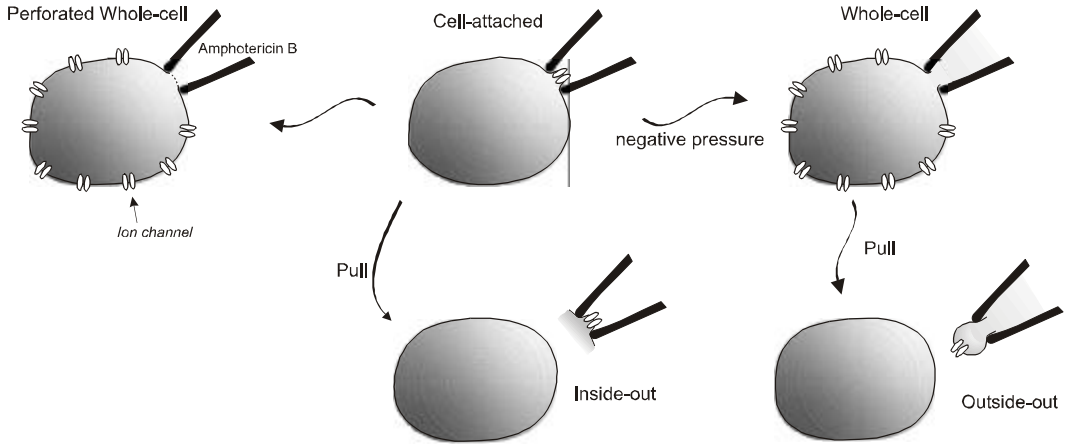

Figure 6. Different patch-clamp configurations used in the thesis. Ion channels that are recorded during each patch-clamp setup are indicated.

Single channel analysis - For analysis of channel currents, records were low-pass filtered at 0.2 kHz, using an 8-pole Bessel filter (Frequency Devices, Haverhill, MA, USA), digitized at 0.8 kHz using a TL-1 DMA interface (Axon Instruments) and stored in a computer. The mean current (I_x) was calculated according to equation 1 using in-house software:

$$I_x = \frac{\sum_{j=1}^{N} (i_j - i_B)}{N} \qquad (1)$$

where S is the number of samples, i_j is the current observed in sample j and i_B is the value of a user-defined baseline. When single channel amplitude (i_x) varied within the same recording, as in paper II, channel activity was expressed as NP_O. The NP_o value was calculated according to equation 2, where mean current (I_x) is divided by the single channel current amplitude (i_x). Open probability (P_o) can also be extracted from equation 2 and provides a relative measurement of the time that a channel spends in the open state. N is the number of channels present in the patch:

$$I_x = N \times P_o \times i_x \qquad (2)$$

Detailed analysis of the dose-response relation for a-ketoisocaproate and ATP (paper II and VI) were performed using acquired data fitted to a modified Hill equation:

$$I / I_c = 1 - \frac{L}{1 + \left(\frac{[\mathrm{X}]}{\mathrm{EC}_{50}} \right)^{-h}} \qquad (3)$$

where [X] is the concentration of the compound, L is the maximal inhibition caused by the compound, EC_{50} is the concentration of [X] causing half-maximal inhibition or stimulation and h is the slope parameter corresponding to the Hill coefficient. The I/I_c represents the relative channel activity, expressed as the ratio of mean current found during (I) and prior (I_c) to the addition of the test compound.

For analysis of single channel kinetics, current records were filtered between 0.2-1.0 kHz and digitized at 0.8-5.0 kHz. Open time kinetics were determined using in-house software and pClamp software (Axon Instruments) by digitizing segments of the current records (10-60 s long) and forming histograms of baseline and open-level data points.

Analysis of the distribution of channel open times was restricted to segments containing no more than three active channels. Events were identified using a 50% amplitude criterion. At the single channel level, the gating kinetics of the K_{ATP} channel for outward currents can be described schematically using a three-state kinetic model (statement 4) consisting of transitions between two open states and one closed state:

$$O_1 \underset{k_{13}}{\overset{k_{31}}{\leftrightarrows}} C_3 \underset{k_{23}}{\overset{k_{32}}{\rightleftarrows}} O_2 \qquad (4)$$

where O are open-states and C_3 closed-state. O_1 and O_2 are short and long openings, respectively. The rate constants (*k*) can be derived by approximation of the single-channel data to exponential components (*m*) of the probability distribution function (equation 5) by the method of maximum likelihood (Colquhoun and Sigworth, 1983):

$$F(t) = \sum_{j=1}^{m} \frac{a_j}{t_j} e^{-t/t_j} \qquad (5)$$

where t_j are the time constants and the coefficients a_j are the areas of each component. The coefficients a_j sum to unity. Single-channel kinetic analysis in paper II, IV and V is presented in figures as open-time histograms, with the optimal fitted exponential function (equation 5) superimposed.

Intracellular Ca^{2+} measurements - For $[Ca^{2+}]_i$ measurements, ß-cells were pre-incubated 20 min before experiments with 1.5 µM fura-2/AM at 37EC. The cells, attached to coverslips, were then transferred to a perfusion chamber and the $[Ca^{2+}]_i$ measurements were performed essentially as previously reported (Nilsson *et al.*, 1987). The microscope was equipped with a photon counting photometer and connected to a SPEX fluorolog-2 CM1T11I system (Spex Industries, Edison, NJ, USA), allowing fluorometry using two excitation wavelengths, 340 and 380 nm. A ratio between 340/380 fluorescence was obtained every second. Using the transformation algorithm as described (Grynkiewicz *et al.*, 1985), fluorescence ratios were converted into $[Ca^{2+}]_i$ values.

Confocal microscope - Oocytes expressing GFP labeled Kir6.2 with and without co-expressing SUR1, were placed in a perfusion chamber mounted on a Leica CLSM (Leica Lasertechnik, Heidelberg, Germany). For the confocal microscope the following settings were used: 10x/0.45 oil Leitz Fluotar objective lens, excitation wavelength 488 nm

(argon/krypton laser) and a 515/20 nm bandpass emission filter. In order to compare the fluorescence intensity, the settings for the photo-multiplier and the pinhole were kept constant and all experiments were performed on the same preparation of oocytes. Fluorescence intensity was calculated using the ISee-software for UNIX (Inovision Corporation, Durham, UK).

Measurements of long-chain CoA ester content and insulin release - Long-chain CoA ester were extracted and measured enzymatically as described previously (Williamson and Corkey, 1979; Corkey, 1988a). In short, cells were extracted in cold 1% (w/v) trichloroacetic acid (TCA) and the TCA supernatant was neutralized with 4 equal volume washes of diethyl ether using vacuum suction to remove the ether. Supernatants were then freeze-dried in a speed-vac (Savant) and finally stored at -80EC until analysis. LC-CoA esters were hydrolyzed to yield free CoA-SH by alkaline hydrolysis at pH 11.5 and 55EC in the presence of 1 mM dithiothreitol. Free CoA-SH content was determined enzymatically using a fluorometric assay in which a-ketoglutarate dehydrogenase catalyzes the conversion of NAD to NADH in the presence of CoA-SH and an excess of a-ketoglutarate.

Insulin was assayed by radioimmunoassay (RIA), using the assay protocol and reagents from Linco Research Inc. (St Louis, MO, USA). Both LC-CoA esters and insulin RIA analysis were performed using clonal pancreatic ß-cells (HIT-T15) between passages 64 and 80. Culture properties for HIT-cells were the same as described for primary culture of mouse pancreatic ß-cells (see *ob/ob mouse*).

Molecular Biology

Vector construction - Plasmid pB.Kir6.2 was created by subcloning the cDNA of mouse Kir6.2 (GenBank accession number D50581) into pBluescript II SK(Stratagene, La Jolla, CA, USA) via *Cla*I and *Eco*RI digestion. To obtain the C-terminally truncated version of mKir6.2_{1-364}, i.e. Kir6.2ΔC26, a stop-codon (TGA) was introduced at the position, which codes for amino acid 365, thus generating pB.Kir6.2ΔC26. For the construction of pB.Kir6.2-GFP a *Nru*I-site was introduced by substituting nucleotides coding for the last amino acid and the stop-codon, i.e. TCC TGA, for TCG CGA. This allowed in-frame fusion of GFP to the C-terminus of Kir6.2. To create pB.GFP-Kir6.2, a *Sma*I site was introduced into the GFP cDNA at the nucleotides coding for Asp235 and Glu237, generating pCMV.GFP0. A *Nru*I-site was introduced into Kir6.2 by substituting nucleotides coding for Ser3 (TCC->TCG) and the *Nru*I-*Xba*I fragment, which contained

the Kir6.2 sequence coding for amino acids 4-390, was inserted in-frame into the *Sma*I-*Xba*I opened pB.GFP0. In pB.SUR1, the cDNA of hamster SUR1 (GenBank accession number L40623) has been subcloned into pBluescript II SK (Stratagene) into the *Eco*RI site. In all plasmids the orientation of the expression cassette has been choosen in a way that transcripts can be obtained by T7 RNA polymerase. All mutations were introduced by employing the QuikChange Mutagenesis Kit (Stratagene). All vector constructions were verified by DNA sequence analysis.

In vito transcription - Plasmids pB.Kir6.2, pB.Kir6.2-GFP, pB.GFP-Kir6.2 and pB.SUR1 were used for *in vitro* transcription. Plasmid DNA was prepared using a QIAprep Spin Miniprep Kit (Qiagen GmbH, Hilden, Germany) and purified using GenePrep Kit (Ambion, Austin, TX, USA). The respective plasmid DNA was linerized by digestion with *Xba*I, purified by phenol-chloroform treatment and thereafter ethanol-precipitated. The DNA pellet was redissolved in water and an aliquot containing 0.5-1 µg DNA was used for *in vitro* transcription. Capped mRNA was synthesized by employing the mMESSAGE mMACHINE T7 Kit (Ambion). The purified mRNA was dissolved in 10 mM Tris-HCl (pH 7.4) and stored in aliquotes at -80°C. The integrity of obtained *in vitro* transcripts was analyzed by formaldehyde-denaturating RNA agarose gel electrophoresis using RNA length marker. The amount of transcripts was determined by ethidium bromide staining. The average yield of transcripts was 20-30 µg per *in vitro* transcription.

Statistics - All data are given as mean ± S.D. or S.E.M, as indicated. If not indicated, experiments were repeated at least three times. Statistical significance was analyzed using paired and unpaired Student's *t*-test or ANOVA for multiple groups, as appropriate. A *p* value less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Oscillations in ß-cell electrical activity is driven by oscillations in K_{ATP} channel activity **(paper I)** - At intermediate glucose concentrations (7-12 mM) the electrical activity of ß-cells consists of oscillations in membrane potential between a plateau potential of . -40 mV, on which action potentials are superimposed, and more negative . -65 mV electrically inactive periods (Henquin and Meissner, 1984). $[Ca^{2+}]_i$ oscillates in synchrony with electrical activity (Santos *et al.*, 1991) and oscillations in $[Ca^{2+}]_i$ and electrical activity correspond to pulsatile insulin release (Tengholm *et al.*, 1992; Gilon *et al.*, 1993; Smith *et al.*, 1995). Amperometric recordings confirm that insulin secretion oscillates in single intact ß-cells at intermediate glucose concentrations (Fig. 7). At higher glucose levels, the pattern of electrical activity is changed into continuous spiking activity. In paper I it is confirmed that electrical activity and $[Ca^{2+}]_i$ oscillate in intact pancreatic ß-cells at a burst interval between 1-4 min at an intermediate glucose concentration (10 mM; Fig. 1, paper I). Inhibition of K_{ATP} channel currents with the sulfonylurea compound tolbutamide clamped the $[Ca^{2+}]_i$ at a sustained elevated level for the duration of tolbutamide stimulation (Fig. 1, paper I). This indicates that these spontaneous oscillations are susceptible to K_{ATP} channel inhibition. Measurements of channel activity in intact ß-cells, using the cell-attached configuration, showed frequent openings of K_{ATP} channels at non-stimulatory glucose concentration (3 mM glucose). As the glucose concentration was raised, channel activity subsided and action potentials were seen. After 1-3 min the action potentials ceased and openings of K_{ATP} channels were again observed (Fig.2, paper I). In contrast to previous suggestions that K_{ATP} channels remain closed during the repolarization phase of glucose-induced oscillations (Smith *et al.*, 1990), these results provide evidence that the mechanism behind the oscillatory electrical activity is spontaneous oscillations in K_{ATP} channel activity. Glucose metabolism is known to oscillate in various systems (Tornheim, 1997). Accordingly, it has been proposed that oscillations in $[Ca^{2+}]_i$ in the pancreatic ß-cell could be caused by fluctuations in the activity of the K_{ATP} channel due to oscillations in metabolism (Corkey *et al.*, 1988b). Our results are consistent with this view. K_{ATP} channel activity increases when ATP/ADP ratio is lowered, which can experimentally be achieved by applying Na-azide (NaN_3), an inhibitor of glucose metabolism (Detimary *et al.*, 1996). At 20 mM glucose, addition of 3 mM NaN_3 increased K_{ATP} channel activity in intact cells, assessed using the perforated-patch technique, and thus, hyperpolarized the ß-cell (Fig. 4, paper I). Further support for this notion is that K_{ATP} channel activity has been shown to oscillate at non-stimulatory glucose concentrations (Dryselius *et al.*, 1994). Due to the low

input resistance under these conditions, oscillations in K_{ATP} channel activity do not produce oscillations in membrane potential that can be resolved by recording of the membrane potential. But with the addition of 10 µM tolbutamide (Fig. 3, paper I), which partially blocks K_{ATP} channel activity, these oscillations in K_{ATP} channel activity are able to depolarize the membrane to the level at which Ca^{2+} channels are activated. Taken together, paper I shows that K_{ATP} channels exhibit spontaneous oscillations in the single ß-cell at intermediate glucose concentrations and that this gives rise to the well-documented oscillations in electrical activity and $[Ca^{2+}]_i$.

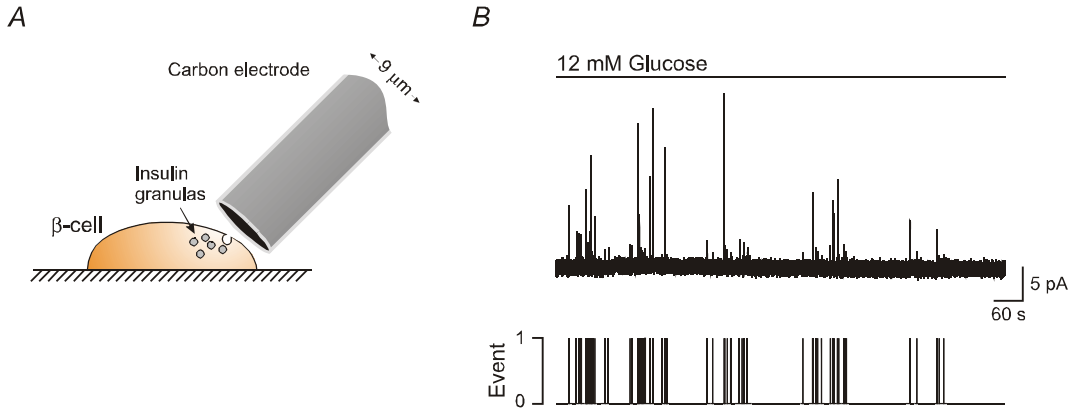

Figure 7. ***A***, schematic illustration of the amperometric technique. Cells were preloaded with serotonin as a marker for insulin release (Aspinwall *et al.*, 1997). ***B***, Amperometric recording from a single pancreatic ß-cell (*Top*). *Bottom*, idealized curve based on the recording above. Unpublished figure (Deeney et al. 1999, *manuscript*).

Direct inhibition of K_{ATP} channel activity by a-ketoisocaproate **(paper II)** - Like glucose, L-leucine is able to potently stimulate insulin release (Panten *et al.*, 1974). As shown in paper II, L-leucine had no direct effect on K_{ATP} channel activity, supporting the notion that L-leucine needs to be metabolized in order to act as an insulin secretagouge. However, an intriguing finding is that a non-metabolizable analogue of L-leucine, ß-2-aminobicyclo[2.2.1] heptane-2-carboxylic acid (BCH), stimulates insulin release, indicating that additional mechanisms may be involved besides metabolism (Panten and Christians, 1973; Sener *et al.*, 1981). In figure 8 it is shown that BCH causes a direct and non-reversible inhibition of the K_{ATP} channel activity, thereby at least partly, explaining the insulinotropic effect of BCH. The deamination product of L-leucine, a-ketoisocaproate, has been extensively used over the last decades since it, like glucose, causes membrane depolarization and a sustained insulin release from pancreatic ß-cells. In contrast to

glucose, a-ketoisocaproate is exclusively metabolized within the mitochondria. The common metabolic signal for glucose and a-ketoisocaproate has been proposed to be mitochondrially-generated ATP which closes the K_{ATP} channel and thus causes membrane depolarization, Ca^{2+} influx and insulin release. However, the results presented in paper II show that a-ketoisocaproate directly and in a dose-dependent manner inhibited the K_{ATP} channel in isolated membrane patches. The concentration of a-ketoisocaproate causing half-maximal reduction of channel activity was 8 mM and not dependent on the presence of adenine nucleotides or intracellular Mg^{2+}. No blocking effect by a-ketoisocaproate could be observed on two other K^+ channels (K_{SK} and K_{BK}) present in the pancreatic ß-cell (Fig. 5, paper II). Much of the proof that a-ketoisocaproate needs to be metabolized in order to stimulate insulin release has been based on the findings that blocking of mitochondrial metabolism omits the insulinotropic effect of a-ketoisocaproate (Hutton *et al.*, 1980). Therefore, we have investigated the effects of a-ketoisocaproate on whole-cell K_{ATP} channel currents in the presence of NaN_3. In the presence of NaN_3, the pancreatic ß-cell was hyperpolarized presumably because a decreased ATP/ADP ratio activates K_{ATP} channels (paper I). Addition of a-ketoisocaproate, in the presence of NaN_3, reduced whole-cell K_{ATP} channel currents by approximately 50% (Fig. 3, paper II), indicating that the effect of a-ketoisocaproate on the K_{ATP} channel is not dependent on metabolism. The K_{ATP} channel activity was not completely blocked in the presence of NaN_3 as required for membrane depolarization (similar to tolbutamide data in Fig. 4*B*, paper I), Ca^{2+} influx and insulin release. Thus, in the presence of NaN_3, a-ketoisocaproate decreased input conductance from 4.0±0.8 nS to 2.1±0.5 nS (n = 5) which is not enough to cause membrane depolarization to the level were action potentials occur (Cook *et al.*, 1988; Ashcroft and Rorsman, 1989). This finding suggest that a-ketoisocaproate closes the K_{ATP} channel directly without involvement of metabolism and explains previous assumption that a-ketoisocaproate had to be metabolized in order to inhibit the K_{ATP} channel. The fact that a-ketoisocaproate depolarized the membrane faster than glucose and was temperature insensitive (Fig. 3*C*, paper II), further suggests that a-ketoisocaproate is able to depolarize the ß-cell and stimulates insulin release independent of metabolism of the ketone acid. Interestingly, it has been shown that a-ketoisocaproate-induced ATP-production reaches a plateau already at micromolar concentrations (Lembert and Idahl, 1998), which is far below the concentration at which a-ketoisocaproate stimulates insulin release (>3.3 mM; Panten *et al.*, 1972; Hutton *et al.*, 1980). Other intermediate substrates in the breakdown of a-ketoisocaproate, like acetate, acetoacetate, citrate, pyruvate, D-ß-hydroxybutyrate, were all without any significant direct effect on K_{ATP} channel activity, when studied in

excised inside-out patches isolated from pancreatic ß-cells. Taken together, data presented in paper II suggest that the insulinotropic effect of a-ketoisocaproate, at least in part, can be explained by a direct inhibition of K_{ATP} channels.

Finally, unpublished experiments suggest that the site at which a-ketoisocaproate inhibits the K_{ATP} channel is located on the regulatory subunit SUR1. Injection of mRNA encoding Kir6.2 alone (Kir6.2? C26) resulted in an ATP-sensitive K^+ current which is not sensitive to a-ketoisocaproate. Co-expression of SUR1, resulted in channel activity which was equally sensitive to a-ketoisocaproate as native pancreatic ß-cell K_{ATP} channels (Fig. 8). This finding further support that the effect of a-ketoisocaproate is not due to a nonspecific block of K^+ channel pores but rather involves a specific interaction with the K_{ATP} channel protein complex.

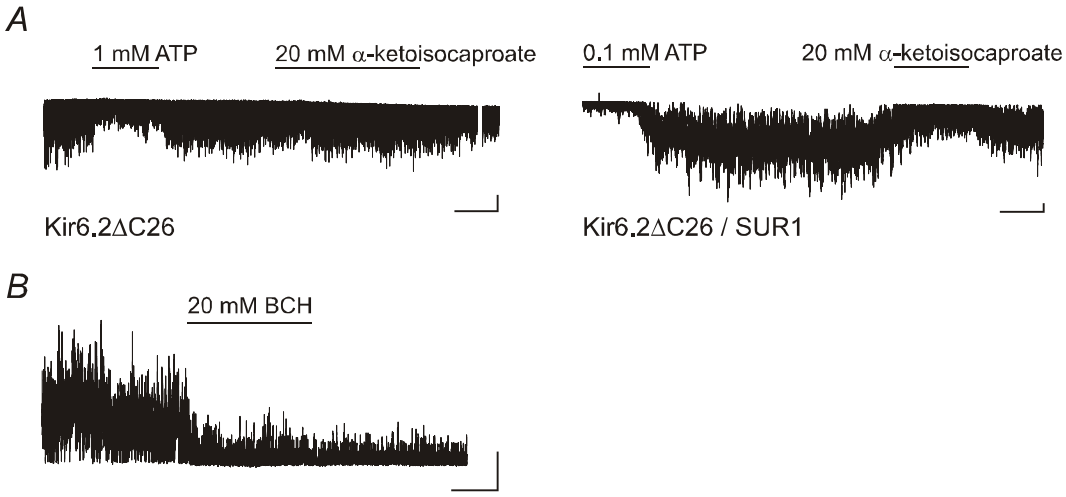

Figure 8. *A*, representative recordings from oocytes injected with mRNA encoding Kir6.2? C26 (*left*) and Kir6.2 togther with SUR1 (*right*). No effect of a-ketoisocaproate could be observed in patches excised from oocytes expressing Kir6.2 alone, whereas a-ketoisocaproate was equally potent inhibiting Kir6.2/SUR1 currents compared to native K_{ATP} channels activity. *B*, addition of 20 mM BCH reduced native ß-cell K_{ATP} channel activity. The effect of BCH was not reversible. Vertical and horizontal calibration bar is 5 pA and 30 s, respectively. (Unpublished observations).

Long-chain acyl CoA esters activate the K_{ATP} channel **(paper III-V)** - In paper III it was established that prolonged exposure of ß-cells to elevated levels of FFA decreased the ability of glucose to stimulate insulin secretion. This observation is consistent with previously published findings in pancreatic islets as well as with *in vivo* studies in animals (Sako and Grill, 1990; Zhou and Grill, 1994) and humans (Paolisso *et al.*, 1995). One possible explanation could be that FFA or metabolic products generated from FFA metabolism, modulate K_{ATP} channel activity, and thereby decrease glucose-induced insulin

secretion. One of these substances could be LC-CoA esters which have been shown to accumulate in response to long-term incubation with elevated FFA (Prentki *et al.*, 1992). Analysis of the total CoA-ester pool, showed an almost 100% increase in total LC-CoA esters following over-night incubation with 0.5 mM palmitate without affecting the levels of short-chain CoA esters (Table 1, paper III). It was also shown that LC-CoA esters are potent and reversible activators of the K_{ATP} channel. The stimulatory effect of LC-CoA esters on K_{ATP} channel activity was dependent on the fatty acid chain length. Effects were seen only with CoA esters of chain length 14-carbons and longer (Fig. 9*C*). No difference in the ability to stimulate channel activity could be seen comparing saturated and unsaturated LC-CoA esters (Fig. 4, paper III) or between *cis*- and *trans*-isoforms of unsaturated acyl-CoA (Fig. 10*I*). The ability of LC-CoA esters to increase K^+ conductance seemed to be specific for the K_{ATP} channel since the esters were without effect on the K_{BK} and K_{SK} channels (Fig. 5, paper III). Other lipids had either inhibitory or no effect on K_{ATP} channel activity (Fig. 10*A*-*C*), well in line with other studies (Fan and Makielski, 1997). LC-CoA esters stimulate channel activity in concentrations ranging between 10-1000 nM (Fig. 1, paper III). In paper IV it was investigated to what extent LC-CoA esters were able to activate the K_{ATP} channel in the presence of fixed ratios of ATP/ADP. At ATP/ADP ratios of 1 and 10, palmitoyl-CoA esters (1 µM) still potently increased channel activity (Fig. 6, paper IV), suggesting that LC-CoA esters are able to activate K_{ATP} channels under more physiological conditions where the channel is controlled by ATP and ADP. A detailed dose-response curve in the presence of an ATP/ADP ratio of 10, resulted in an EC_{50}-value of 0.59±0.11 µM and a Hill-value (*h*) of -2.9±0.8 (*n* = 6-15; Fig. 9).

The cytosolic free concentration of LC-CoA esters is not known for any tissue, but the total intracellular pool of LC-CoA esters has been reported to be in the range of 5-160 µM, depending on the tissue and metabolic state (reviewed by Faergeman and Knudsen, 1997). The vast majority of studies on LC-CoA ester distribution has been performed in liver cells. In ß-cells, the total amount of LC-CoA esters in nmol/g of protein presented in table 1 (paper III) can be converted into µM, extrapolating from liver cell data assuming that the cytosolic water space is 2 ml/g of protein and that the cytosol constitutes ~78% of the total cellular content of LC-CoA esters (Deeney *et al.*, 1992). Calculations based on these assumptions and the measured concentration of LC-CoA esters in the ß-cells, yield a total cytosolic concentration of LC-CoA esters of 76±10 µM under control conditions and 158±15 µM after 18-h incubation with 0.5 mM palmitate. Cytosolic LC-

CoA esters are buffered by acyl-CoA ester bindning protein (ACBP) and fatty acid binding protein (FABP) as well as by partitioning in various membranes. The concentration of cytosolic free LC-CoA esters will depend on the total binding capacity of the cytosol. Clonal pancreatic ß-cells have been reported to have ~520 nmol/g protein (260 µM) of LC-CoA ester binding sites with a K_D-value of ~1 µM (Deeney *et al.*, 1992). A calculation based on these assumptions yields a cytosolic free concentration of LC-CoA esters of 0.39 µM under control conditions and 1.47 µM after incubation with FFA (0.5 mM palmitate for 18-h). It should be noted that some of the remaining 75.6 µM (control) and 156.5 µM (FFA-treated) LC-CoA esters that is bound may be bound to proteins of interest such as the K_{ATP} channel. Although these calculations are crude and relay on several rough estimates, they suggest that the levels of cytosolic free LC-CoA esters present in pancreatic ß-cells may be of physiological importance to regulate the ß-cell K_{ATP} channel.

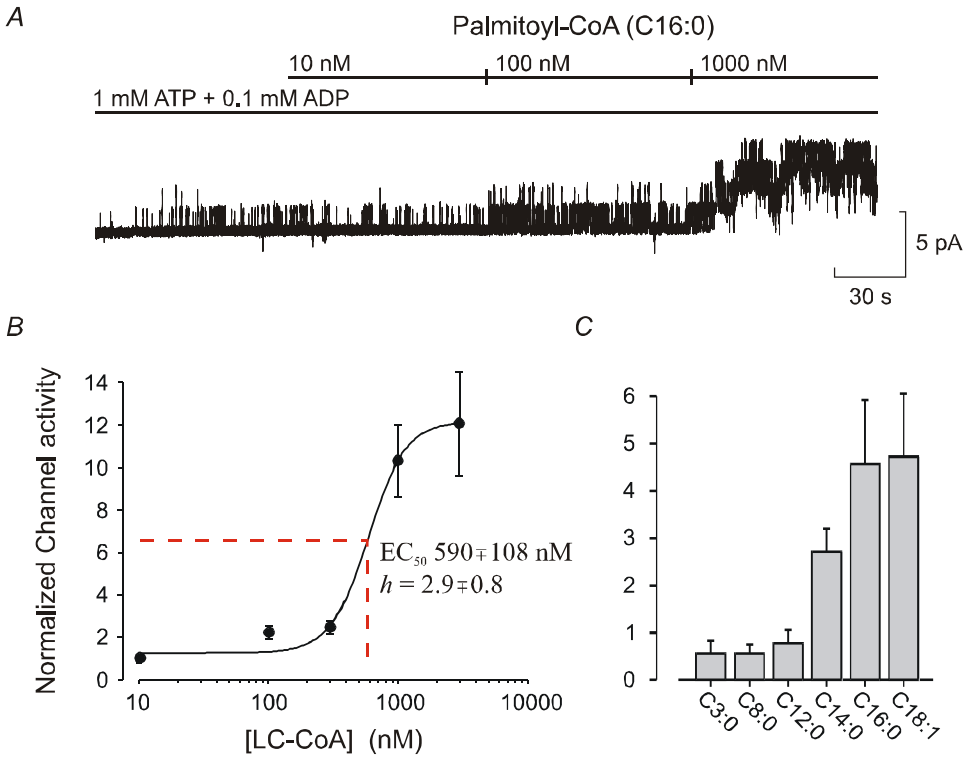

Figure 9. ***A***, K_{ATP} channel recording from an inside-out patch isolated from a mouse pancreatic ß-cell. After addition of ATP/ADP (ratio 10), palmitoyl-CoA ester was added at increasing concentrations. ***B***, Dose-response relationship of LC-CoA esters in the presence of ATP/ADP (ratio 10) on K_{ATP} channel activity. Line was fitted to Hill-equation (Equ. 3). Channel activity was normalized to the current observed in the presence of ATP/ADP in the absence of LC-CoA esters. ***C***, dependence of acyl chain length of the CoA esters. All CoA-esters were added to the intracellular solution at a final concentration of 1 µM.

In freshly isolated patches from pancreatic ß-cells, K_{ATP} channel activity displays open time kinetics which can be described by a single time constant (t) of 10-30 ms (Bokvist *et al.*, 1990; Larsson *et al.*, 1993). Exposure to channel activators, like MgADP and diazoxide, are known to affect open time kinetics by introducing a second time constant (t_2) of 100-200 ms (Larsson *et al.*, 1993), i.e. two distinct openings of long and short duration can be seen. In paper IV, it was shown that the effect of LC-CoA ester mimics the effect of MgADP, not only increasing the channel current, but also by introducing a second open state with a time constant of >200 ms (Fig. 1, paper IV). In a series of experiments using different protocols, it was stipulated that LC-CoA esters are interacting at a binding site separate from that of ATP and MgADP. In short this was based on the findings that; *i*) modulating the K_{ATP} channel using trypsin altered the sensitivity for ATP and MgADP whereas the effect of LC-CoA ester remained virtually unchanged, *ii*) activation by LC-CoA esters did not require the presence of millimolar concentration of Mg^{2+} as ADP does, and finally, *iii*) simultaneous addition of LC-CoA ester and MgADP resulted in a supra-additive effect on channel activity.

The notion that LC-CoA esters have a unique binding site on the K_{ATP} channel complex was further verified in paper V. By expressing subunits of the K_{ATP} channel, Kir6.2 separate and together with SUR1 in *Xenopus* oocytes, it was shown that LC-CoA esters potently activated the pore forming subunit (Kir6.2) of the K_{ATP} channel. These studies were performed using the C-terminally truncated Kir6.2 (Kir6.2? C26) which produces ATP-sensitive K^+ currents in the absence of SUR1 (Tucker *et al.*, 1997). Co-expression with SUR1 did not significantly enhance the stimulatory effect of the esters. The effect of LC-CoA esters on Kir6.2 had the same properties and specificity as shown for the native K_{ATP} channel in paper III and IV, with regard to chain length and channel kinetic characteristics as well as the ability to counteract the inhibitory effect of ATP. These results suggest that K_{ATP} channel activation by LC-CoA esters is mediated either by a direct interaction with Kir6.2 subunit or, possibly, via a third subunit endogenously expressed in both *Xenopus* oocytes and pancreatic ß-cells.

Studies in cardiac myocytes have demonstrated that all elements in the PKC signal transduction system are available in excised membrane patches and able to interact effectively (Hu *et al.*, 1999). However, PKC, which has been shown to affect K_{ATP} channel activity in pancreatic ß-cells (Wollheim *et al.*, 1988), is probably not involved since LC-CoA esters were still able to activate the channel in the absence of ATP and Mg^{2+}, which are required for PKC function. Moreover, in the presence of the highly specific and potent PKC inhibitor bisindolylmaleimide (BIM), LC-CoA esters were still

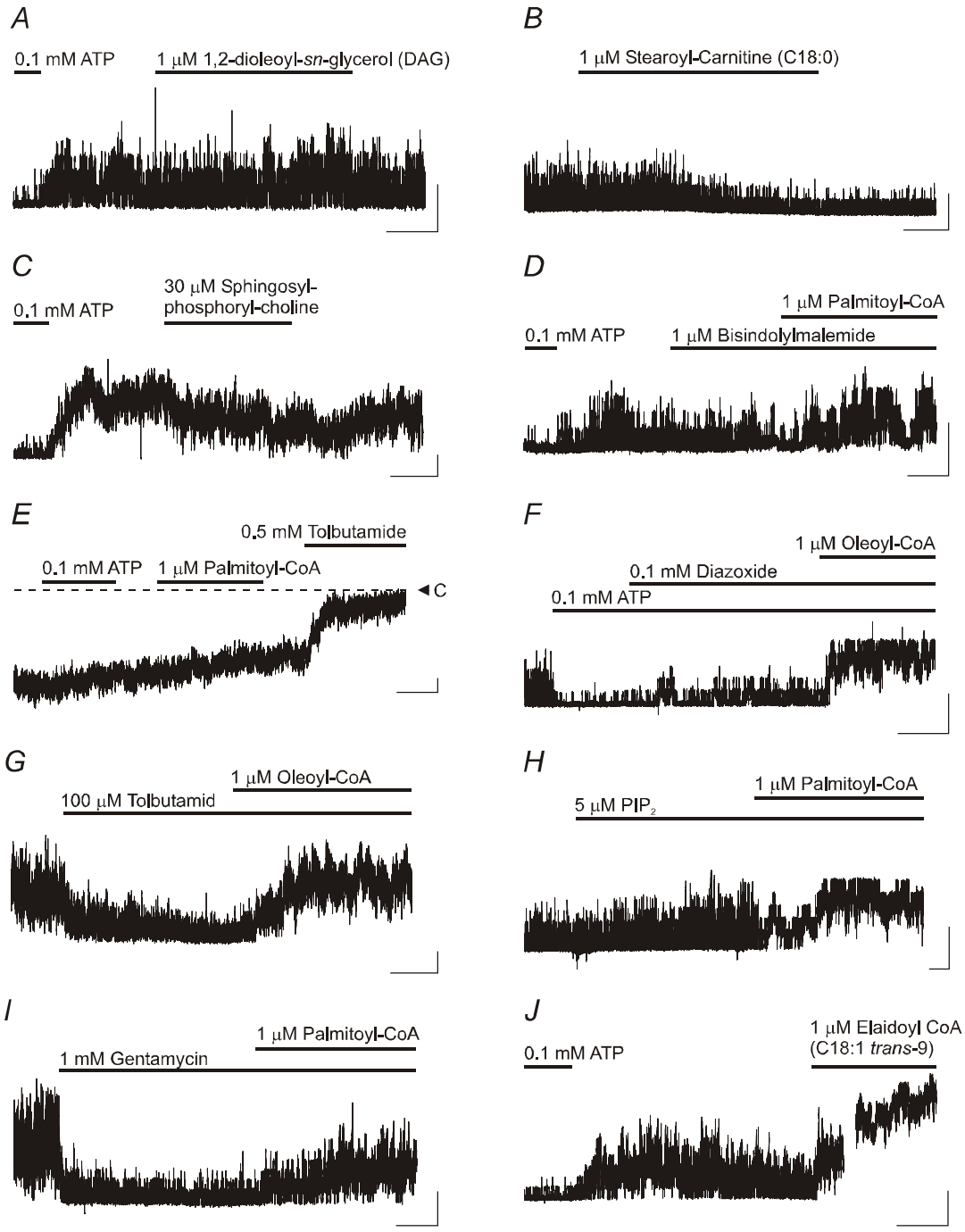

Figure 10. Summary of effects of different lipids and LC-CoA esters on pancreatic ß-cell K_{ATP} channel activity. ***A*-*C***, effects of different complex lipids on the K_{ATP} channel activity. ***D***, presence of PKC inhibitor BIM did not abolish channel activation by palmitoyl-CoA ester. ***E***, Palmitoyl-CoA ester did not activate the K_{ATP} channel when applied to the outside of the plasma membrane, nor did diazoxide, tolbutamide, PIP_2 or gentamycin prevent LC-CoA ester-induced channel activation (***F*-*I***). ***J***, Altering from *cis*- to *trans*-position of the unsaturated lipid, did not affect the ability of LC-CoA esters (C18:1 *trans*-9) to activate the channel. Vertical and horizontal calibration bars in each recording symbolize 5 pA and 30 s, respectively.

able to activate channel activity (Fig. 10*D*), further supporting the notion that PKC is not involved. Although the results do not allow us to conclude that LC-CoA esters interact directly with Kir6.2 subunit itself, the data presented strongly favors such an interpretation.

The anionic phospholipids PIP and PIP_2 interact and enhance the activity of the native Kir channel superfamily, including cardiac and ß-cell K_{ATP} channels (Hilgemann and Ball, 1996; Fan and Makielski, 1997; Fig. 10*H*). Interaction with the C-terminus of the Kir6.2 subunit has been proposed to be the mechanism behind the stimulatory effect of these phospholipids on K_{ATP} channels (Fan and Makielski, 1997; Shyng and Nichols, 1998). LC-CoA esters resemble structurally phospholipids in that both substances have a charged head-group and a lipid tail. However, the properties of PIP/PIP_2 activation are not identical to those of LC-CoA esters; *i*) the reported concentration of PIP_2 that activates the K_{ATP} channel is approximately 10-100 times higher that of LC-CoA. *ii*) PIP_2 activates both Kir6.2 and Kir1.1a currents (Fan and Makielski, 1997), a related Kir channel that has ~45% amino acid sequence similarity with Kir6.2, whereas LC-CoA esters only activates Kir6.2 (Gribble *et al.*, 1998). *iii*) the stimulatory effects of PIP/PIP_2 can be dramatically reduced by adding polyvalent cation antibiotic gentamycin or neomycin (thought to abolish the stimulatory effect of PIP/PIP_2 by chelating negative charges in the anionic head of the phospholipids) to the inner membrane surface (Fan and Makielski, 1997), a reduction that could not be observed for LC-CoA esters (Fig. 10*I*), and finally *iv*) unpublished findings show that LC-CoA esters can still activate the K_{ATP} channel in the presence of PIP_2 (Fig. 10*H*). Taken together, these findings make it unlikely that PIP/PIP_2 and LC-CoA ester interact with the channel by common mechanisms. Further support of this notion is the finding that Kir6.1, which is thought to be the pore forming subunit in K_{ATP} channels found in the mitochondria (Suzuki *et al.*, 1997b), is inhibited by palmitoyl- and oleoyl-CoA esters (Paucek *et al.*, 1996) yet activated by PIP/PIP_2.

Although the general concensus is that K_{ATP} channel activity is regulated by alterations in the ATP/ADP ratio, some intriguing findings suggest that this might not be the whole story. Whole-cell K_{ATP} channel current increases almost 40-fold in response to metabolic inhibitors (Ashcroft and Gribble, 1998). A similar fold of increase in channel activity can not be obtained in excised patches scanning through different ATP/ADP ratios (own unpublished observations). Mutations in the NBFs of SUR1 alter the sensitivity to MgADP, but metabolic activation is not affected to the same extent (Nichols *et al.*, 1996; Gribble *et al.*, 1997). Taken together, these observations suggest that additional substances are involved in K_{ATP} channel regulation. One such substance could very well be LC-CoA

esters, which we show are potent endogenous direct activators of the K_{ATP} channel. If LC-CoA esters are able to affect the K_{ATP} channel in intact cells still remains to be clarified. Preliminary studies performed in human pancreatic ß-cells (Fig. 11 *left*), confirm that LC-CoA esters are potent activators of the human pancreatic K_{ATP} channel, and furthermore, show that channel activity increases in ß-cells following exposure to elevated cytosolic LC-CoA esters using the whole-cell configuration (Fig. 11 *right*).

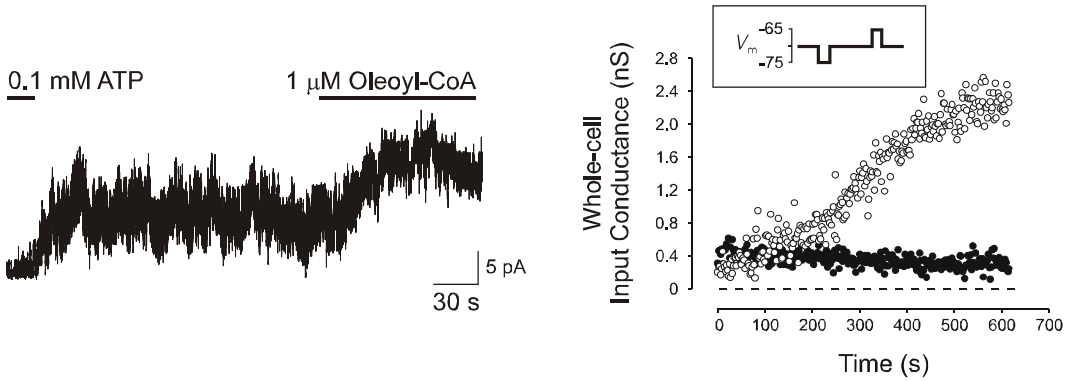

Figure 11. Effects of LC-CoA esters on human ß-cell K_{ATP} channel. *Left*, in inside-out patches K_{ATP} channel activity increased significantly after addition of oleoyl-CoA ester. *Right*, inclusion of 1 μM palmitoyl-CoA ester in the pipette solution containing 1 mM ATP and 0.1 mM Mg-ADP, dramatically increased the whole-cell K^+ current (open circles), whereas a gradual decrease was observed in the absence of the ester (closed circles). Whole-cell currents were measured as input conductance in human pancreatic ß-cells, by voltage-clamping the cell at -70±5 mV (200 ms; *inset*).

Based on the findings presented in this thesis it is likely that LC-CoA esters indeed have an endogenous binding site located on the pore forming subunit of the K_{ATP} channel (Fig. 12). Future investigations will try to identify the amino acid sequence responsible for LC-CoA ester binding and use that information to find the LC-CoA ester binding motif. Preliminary studies show that Kir6.2 has several possible regions with high sequence similarity with other known LC-CoA esters binding proteins such as long-chain CoA ester binding protein (ACBP), adenine nucleotide translocase (ANT) and fatty acid synthethase. Point mutations within these regions severely decrease the potency of LC-CoA esters.

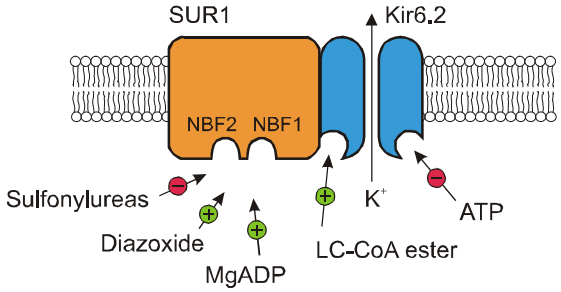

Figure 12. Highly schematic figure of the K_{ATP} channel (Kir6.2 and SUR1). ATP and LC-CoA esters interact at separate binding sites at the Kir6.2, whereas MgADP, diazoxide and sulfonylureas modulate K_{ATP} channel activity via the SUR1 subunit.

Separate actions of SUR1 on K_{ATP} channel regulation and assembly **(paper VI)** - The inability of Kir6.2 subunits to function as K^+ channels in the absence of SUR1 (Inagaki *et al.*, 1995a) has been suggested to be due to trafficking failure and that SUR1 acts as a chaperone to permit Kir6.2 subunits to form tetramers and/or to traffic to the plasma membrane (Tucker *et al.*, 1997; Clement *et al.*, 1997). However, utilizing green fluorescent protein (GFP) labelling of Kir6.2 and SUR1 *in vivo* in mammalian cells indicates that both Kir6.2 and SUR1 can independently traffic toward the cell membrane (Makhina and Nichols, 1998). The authors also suggest that following co-expression of both subunits, a physical interaction between SUR1 and Kir6.2 occurs in the endoplasmic reticulum which either facilitates insertion of channel-containing vesicles into the plasma membrane or stabilizes channels once inserted in the membrane.

In paper VI it is shown that SUR1-induced increase in number of active channels in the oocyte plasma membrane is independent of positioning of the GFP-tag on either the C- or N-terminus of Kir6.2 (Fig. 13). Co-expression with SUR1 did not alter the amount of membrane GFP-fluorescence, indicating that SUR1 is not involved in Kir6.2 transport to the oocyte membrane (Fig. 1, paper VI). Hence, increase in channel activity can not be explained by an increased number of Kir6.2 copies in the plasma membrane. The increase in current can in principal depend on either; inability of Kir6.2 to form tetramers in the absence of SUR1 (i.e. inactive Kir6.2 monomers in the plasma membrane) or SUR1 increases the open probability (P_O) of Kir6.2. The latter has been shown experimentally (Babenko *et al.*, 1999). Available data do not allow us to differentiate between these two possibilities. The ability of SUR1 to convey its modulatory functions on channel activity was only observed when GFP was tagged to the C-terminus of Kir6.2 (Kir6.2-GFP).

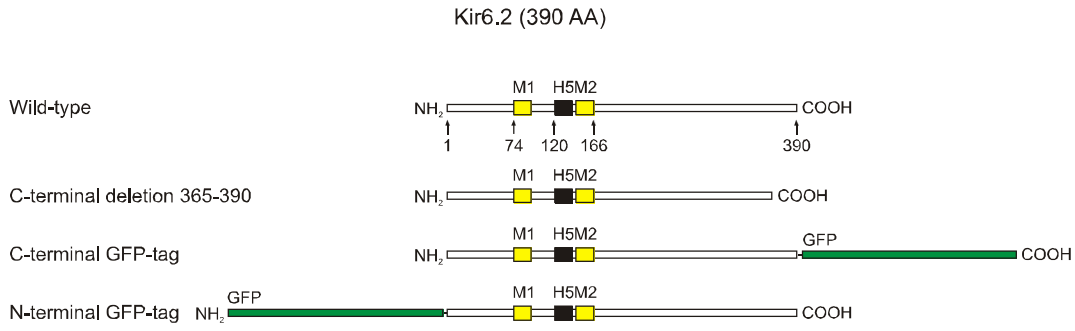

Figure 13. Schematic diagram of the Kir6.2 subunit used in paper V and VI, illustrating the regions in the C- and N-termini that were deleted or fused with GFP. Kir6.2 contains two transmembrane segments (M1 and M2) and a pore-forming H5 segment with high amino acid similarity with other inwardly rectifying K^+ channels (Inagaki *et al.*, 1995a; Minor *et al.*, 1999).

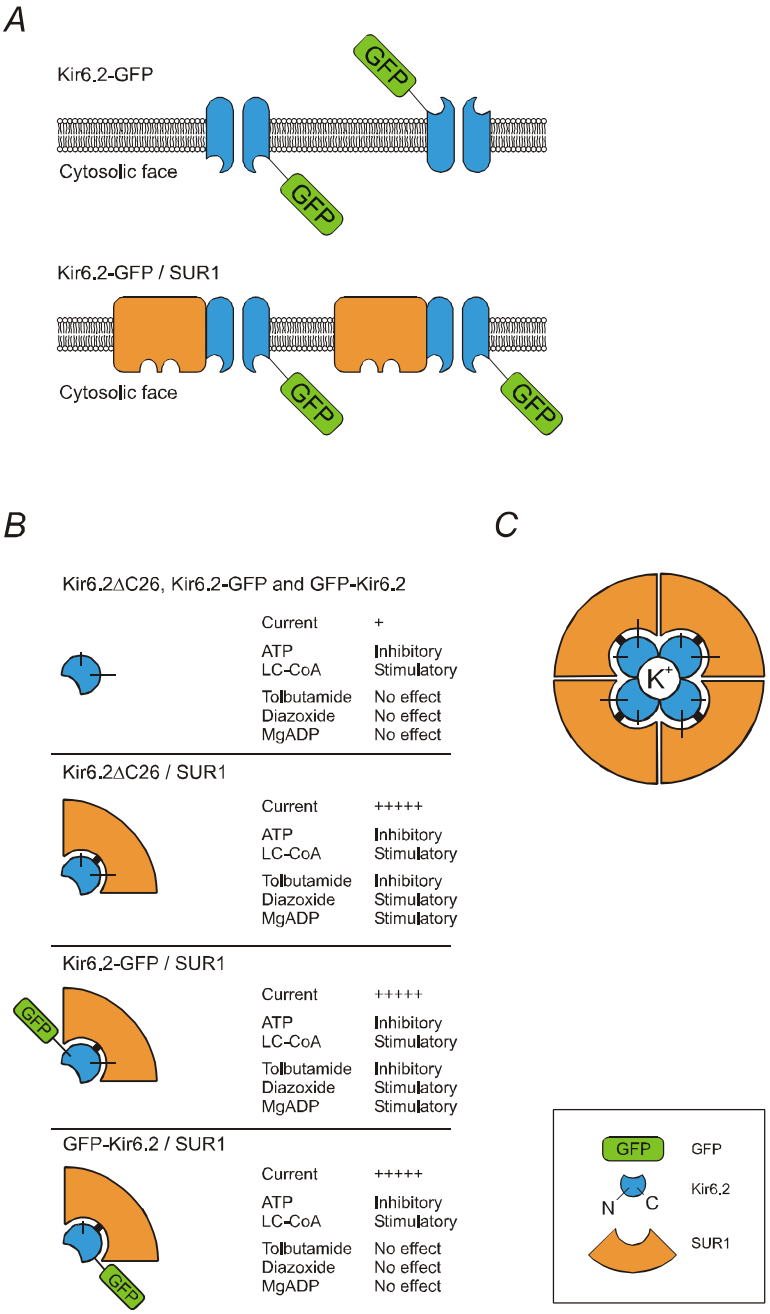

Figure 14. Schematic model of the interactions between K_{ATP} channel subunits. ***A***, expression of Kir6.2-GFP alone resulted in a population of Kir6.2 channels which were malinserted into the plasma membrane, out-out orientation. Malinserted channels were not observed following co-expression of SUR1. ***B***, summary of different parameters on different Kir6.2 clones, expressed in the presence and absence of SUR1. The data favor a model were Kir6.2 and SUR1 interacts at least at two distinct domains. One of those constitutes the N-terminal, since fusion of GFP to the N-terminal (*bottom*) results in channel activity which is not sensitive to agents acting via SUR1 (tolbutamide, diazoxide and MgADP). In contrast, neither N- nor C-termini are involved in Kir6.2 orientation and increased number of active channels. ATP and LC-CoA esters were in all constructs able to exert their effects. ***C***, model of Kir6.2 and SUR1 interactions in the octameric complex.

However, neither tolbutamide nor MgADP/diazoxide were able to inhibit or stimulate GFP-Kir6.2/SUR1 (N-terminal GFP on Kir6.2) channel activity, respectively. These observations suggest that the N-terminus participates as a 'signalling link' between SUR1 and Kir6.2 by which SUR1 modulates channel activity. Our data indicate that SUR1 has multiple and distinct roles in the formation of functional K_{ATP} channels. Although recruitment of Kir6.2 subunits by SUR1 has recently been suggested to explain the increase in functional K_{ATP} channels (Makhina and Nichols, 1998), our data shows that SUR1-promoted increase in channel activity is independent from recruitment. By using GFP-tagged Kir6.2 chimeras we could show that SUR1 promoted a correct orientation of Kir6.2 in the membrane (Fig. 14*A*). The data also constitute direct proof of the "in-in" orientation of Kir6.2 in the K_{ATP} channel complex, earlier only suggested from functional studies. Furthermore, by employing GFP as a tool to disturb proper interaction between Kir6.2 and SUR1, we show that the ability of SUR1 to increase number of active channels and to convey sensitivity towards sulfonylurea and Mg^{2+} nucleotides involves distinct mechanisms in the Kir6.2 and SUR1 interaction. Whereas the position of the GFP-tag had no influence on SUR1-induced increase in number of active channels, the GFP-tag fused to the N-terminus of Kir6.2 drastically disturbed channel modulation by ATP, MgADP and tolbutamide (Fig. 14*B*). This implies that a freely accessible N-terminus of Kir6.2 is required for SUR1 to convey its modulatory functions, whereas no direct accessibility to either N- or C-termini is needed to obtain electrically active K_{ATP} channels. In conclusion, our data indicate that the nature of the interaction between SUR1 and Kir6.2 involves several distinct mechanisms, where SUR1-induced organization of Kir6.2 and the ability of SUR1 to convey sensitivity towards for example ADP and sulfonylurea are two such mechanisms (Fig. 14*B*-*C*).

CONCLUSION

1. K_{ATP} channels oscillate spontaneously in intact ß-cells subsequent to glucose stimulation which gives rise to oscillations in electrical activity and $[Ca^{2+}]_i$. Oscillations in K_{ATP} channel activity is likely to be due to oscillations in metabolites derived from glucose metabolism.

2. Detailed patch-clamp studies in isolated membrane patches and intact ß-cells show that a-ketoisocaproate reversibly and in a dose-dependent manner directly inhibits the K_{ATP} channel. Since a-ketoisocaproate has been used as a tool studying the relative role of oxidative phosphorylation in the ß-cell, this finding must now be considered in future experimental setups using a-ketoisocaproate as a fuel secretagouge.

3. LC-CoA esters potently activate the K_{ATP} channel. Similar results were obtained in K_{ATP} channel originating from different species including human, mouse, rat primary cells as well as from various pancreatic ß-cell lines. Thus, LC-CoA esters form a new class of substances which potently activate K_{ATP} channels. Detailed electrophysiological studies on native and cloned K_{ATP} channels support the view that LC-CoA esters interact at the K_{ATP} channel via a unique binding site, located at the pore-forming subunit Kir6.2. Kir6.2 is expressed in a number of tissues including heart, skeletal muscle and certain brain regions and is believed to serve as the pore-forming subunit in all these tissues. LC-CoA esters may therefore also influence K_{ATP} channel function in tissues other than the ß-cell.

4. An intact N-terminus of Kir6.2 is necessary for SUR1 to mediate its effects on K_{ATP} channel activity, possibly by a mechanism distinct from the one underlying alterations in gating and the apparent decrease in ATP sensitivity. Thus, SUR1 promotes the formation of functional K_{ATP} channels by interacting with Kir6.2 at more than one domain thereby guaranteeing a correct “in-in” orientation of Kir6.2 in the K_{ATP} channel complex.

ACKNOWLEDGMENT

This work has been performed at the Rolf Luft Center for Diabetes Research, Department of Molecular Medicine at the Karolinska Institute. I wish to express my sincere gratitude and appreciation to **everyone at the Department of Endocrinology and the Rolf Luft Center for Diabetes Research** who helped me during this period. In particular I would like to thank:

Olof Larsson, my supervisor, for introducing, teaching, and helping me understand the field of electrophysiology and ion channels. But most of all for his patience and willingness to discuss science as well as other topics at all time. It has been a privilege to work with Olof for the last years.

Per-Olof Berggren, my co-supervisor, for giving me the opportunity to obtain my research education at the Karolinska. PO's never ending appetite for progress in science has created a fruitful environment.

Annika Lindgren, Pontus Lindqvist, Shao-Nian Yang and Chris Barker for all the great times at the electrophysiology section of Rolf Luft Center for Diabetes Research. Especially I would like to thank Annika for her technical assistance and great practical knowledge in laboratory work, and Chris for reading the thesis manuscript.

Rolf Luft for creating the Rolf Luft Center for Diabetes Research.

Hans Löw, for his never ending enthusiasm for science and scientific work.

Barbara E. Corkey and Jude T. Deeney, Boston University, for allowing me to come and work in Boston, and for long and helpful discussions. A special thanks to Jude for providing me with home during my stay in Boston, and to Barbara for reading parts of the thesis manuscipt.

Suad Efendic and Anna-Lena Hulting for help, interest and support.

Co-authors not mentioned above including **Henrik Kindmark, Bertil Fredholm, Tilo Moede, Barbara and Ingo Leibiger.** I would specially like to thank Tilo Moede, Barbara and Ingo Leibiger for performing the molecular biology procedures.

Tomas Brundin, John Wahren, Eva Selldén and everyone at the department of Clinical Physiology, for introducing me to the world of science and clinical work.

Per Hellström, department of medicine, for introducing me for Olof and PO, and for his enthusiasm for a variety of projects.

Fellow PhD-students **Sofie Strömberg, Jon Tsai, Marie-Louise Håkansson, Håkan Carlqvist and Karin Hehenberger** for all PhD-dinners and creating a good working atmosphere.

I would like to thank **Åsa**, especially for her never-falling patience, support and love. My dear family and all relatives, friends and colleagues who gave me their encouragement and enthusiastic support throughout these years.

This study was supported by grants from the Swedish Medical Research Council (03x-09891, 03x-12549, 03x-09890, 03xs-12708, 19x-00034), the Swedish Diabetes Association, Swedish Society of Medical Research, the Nordic Insulin Foundation Committee, funds of the Karolinska Institute, Sixten Gemzéus Foundation, Berth von Kantzows Foundation, the Juvenile Diabetes Foundation, Magnus Bergvalls foundation and the U.S. Public Health Service Grants DK35914 and DK50662.

REFERENCES

Aguilar-Bryan, L., Clement, J.P., Gonzalez, G., Kunjilwar, K., Babenko, A., and Bryan, J. (1998). Toward Understanding the Assembly and Structure of K_{ATP} Channels. *Physiol. Rev.* **78;** 227-245.

Aguilar-Bryan, L., Nichols, C.G., Wechsler, S.W., Clement, J.P.4., Boyd, A.E.3., Gonzalez, G., Herrera-Sosa, H., Nguy, K., Bryan, J., and Nelson, D.A. (1995). Cloning of the beta cell high-affinity sulfonylurea receptor: a regulator of insulin secretion. *Science* **268;** 423-426.

Alekseev, A.E., Kennedy, M.E., Navarro, B., and Terzic, A. (1997). Burst Kinetics of Co-expressed Kir6.2/SUR1 Clones: Comparison of Recombinant with Native ATP-sensitive K^+ Channel Behavior. *J. Membr. Biol.* **159;** 161-168.

Arkhammar, P., Nilsson, T., Rorsman, P., and Berggren, P.O. (1987). Inhibition of ATP-regulated K^+ channels precedes depolarization- induced increase in cytoplasmic free Ca^{2+} concentration in pancreatic beta-cells. *J. Biol. Chem.* **262;** 5448-5454.

Ashcroft, F.M. (1988). Adenosine 5'-triphosphate-sensitive potassium channels. *Ann. Rev. Neurosci.***11;** 97-118.

Ashcroft, F.M., Ashcroft, S.J., and Harrison, D.E. (1987a). Effects of 2-ketoisocaproate on insulin release and single potassium channel activity in dispersed rat pancreatic beta-cells. *J. Physiol.* **385;** 517-529.

Ashcroft, F.M. and Gribble, F.M. (1998). Correlating structure and function in ATP-sensitive K^+ channels. *Trends Neurosci.* **21;** 288-294.

Ashcroft, F.M., Kakei, M., Kelly, R.P., and Sutton, R. (1987b). ATP-sensitive K^+ channels in human isolated pancreatic B-cells. *FEBS Lett.* **215;** 9-12.

Ashcroft, F.M. and Rorsman, P. (1989). Electrophysiology of the pancreatic beta-cell. *Prog. Biophys. Mol. Biol.* **54;** 87-143.

Ashcroft, F.M. and Rorsman, P. (1990). ATP-sensitive K+ channels: a link between B-cell metabolism and insulin secretion. *Biochem. Soc. Trans* **18;** 109-111.

Ashcroft, S.J. and Ashcroft, F.M. (1990). Properties and functions of ATP-sensitive K-channels. *Cell. Signal.* **2;** 197-214.

Ashford, M.L.J., Sturgess, N.C., Trout, N.J., Gardner, N.J., and Hales, C.N. (1988). Adenosine-5´triphosphate-sensitive ion channels in neontal rat cultured central neurons. *Pflugers Arch.* **412;** 297-304.

Aspinwall, C.A., Brooks, S.A., Kennedy, R.T., and Lakey, J.T. (1997). Effects of intravesicular H^+ and extracellular H^+ and Zn^{2+} on insulin secretion in pancreatic beta cells. *J. Biol. Chem.* **272;** 31308-31314.

Babenko, A., Gonzalez, G., Aguilar-Bryan, L., and Bryan, J. (1999). Sulfonylurea receptor set the maximal open probability, ATP sensitivity and plasma membrane density of K_{ATP} channels. *FEBS Lett.* **445;** 131-136.

Baukrowitz, T., Schulte, U., Oliver, D., Herlitze, S., Krauter, T., Tucker, S.J., Ruppersberg, J.P., and Fakler, B. (1998). PIP_2 and PIP as Determinants for ATP Inhibition of K_{ATP} Channels. *Science* **282;** 1141-1144.

Berggren, P.-O. and Larsson, O. (1994). Ca^{2+} and pancreatic B-cell function. *Biochem. Soc. Trans.* **22;** 12-18.

Bernardi, H., De Weille, J.R., Epelbaum, J., Mourre, C., Amoroso, S., Slama, A., Fosset, M., and Lazdunski, M. (1993). ATP-modulated K^+ channels sensitive to antidiabetic sulfonylureas are present in adenohypophysis and are involved in growth hormone release. *Proc. Natl. Acad. Sci. U.S.A.* **90;** 1340-1344.

Bokvist, K., Rorsman, P., and Smith, P.A. (1990). Block of ATP-regulated and Ca2(+)-activated K^+ channels in mouse pancreatic beta-cells by external tetraethyl-ammonium and quinine. *J. Physiol.* **423;** 327-342.

Bray, K.M. and Quast, U. (1992). A specific binding site for K^+ channel openers in rat aorta. *J. Biol. Chem.* **267;** 11689-11692.

Chou, H.F. and Ipp, E. (1990). Pulsatile insulin secretion in isolated rat islets. *Diabetes* **39;** 112-117.

Chutkow, W.A., Simon, M.C., Beau, M.M.L., and Burant, C.F. (1996). Cloning, tissue expression, and chromosomal localization of SUR2, the putative drug-binding subunit of cardiac, skeletal muscle, and vascular K_{ATP} channels. *Diabetes* **45;** 1439-1445.

Clement, J.P., Kunjilwar, K., Gonzalez, G., Schwanstecher, M., Panten, U., Aguilar-Bryan, L., and Bryan, J. (1997). Association and stoichiometry of K(ATP) channel subunits. *Neuron* **18;** 827-838.

Colquhoun, D. and Sigworth, F.J. (1995). Fitting and Statistical Analysis of Single-Channel Records. In: *Single-Channel Recording*, 2nd Ed.,483-585. Edited by Sakmann, B. and Neher, E., New York, Plenum Press.

Cook, D.L. and Hales, C.N. (1984). Intracellular ATP directly blocks K+ channels in pancreatic B- cells. *Nature* **311;** 271-273.

Cook, D.L., Satin, L.S., Ashford, M.L., and Hales, C.N. (1988). ATP-sensitive K+ channels in pancreatic beta-cells. Spare-channel hypothesis. *Diabetes* **37;** 495-498.

Corkey, B.E. (1988a) Analysis of acyl-coenzyme A esters in biological samples. In: *Methods in enzymology*, 55-70. AnonymousAcademic Press, Inc.

Corkey, B.E., Deeney, J.T., Glennon, M.C., Matschinsky, F.M., and Prentki, M. (1988b). Regulation of Steady-state Free Ca^{2+} Levels by the ATP/ADP Ratio and Orthophosphate in Permeabilized RINm5F Insulinoma Cells. *J. Biol. Chem.* **263;** 4247-4253.

Davis, N.W., Standen, N.B., and Stanfield, P.R. (1991). ATP-dependent potassium channels of muscle cells: their properties, regulation, and possible functions. *J. Bioenerg. Biomembr.* **23;** 509-535.

Dean, P.M. and Matthews, E.K. (1968). Electrical activity in pancreatic islet cells. *Nature* **219;** 389-390.

Denney, J.T., Bränström, R., Corkey, B.E., Larsson, O., and Berggren, P.-O. (1999). The Use of ^{3}H-Serotonin as a Marker for Insulin Release in Insulin-Secreting Cells (INS-1). *Manuscript.*

Deeney, J.T., Tornheim, K., Korchak, H.M., Prentki, M., and Corkey, B.E. (1992). Acyl-CoA esters modulate intracellular Ca^{2+} handling by permeabilized clonal panreatic β-cells. *J. Biol. Chem.* **267;** 19840-19845.

Detimary, P., Berghe, G.V.D., and Henquin, J.C. (1996). Concentration dependence and time course of the effects of glucose on adenine and guanine nucleotides in mouse pancreatic islets. *J. Biol. Chem.* **271;** 20559-20565.

Dryselius, S., Lund, P.E., Gylfe, E., and Hellman, B. (1994). Variations in ATP-sensitive K^+ channel activity provide evidence for inherent metabolic oscillations in pancreatic beta-cells. *Biochem. Biophys. Res. Comm.* **205;** 880-885.

Dunne, M.J., Findlay, I., and Petersen, O.H. (1988). Effects of pyridine nucleotides on the gating of ATP-sensitive potassium channels in insulin-secreting cells. *J. Membr. Biol.* **102;** 205-216.

Dunne, M.J. and Petersen, O.H. (1986a). GTP and GDP activation of K^+ channels that can be inhibited by ATP. *Pflugers Arch.* **407;** 564-565.

Dunne, M.J. and Petersen, O.H. (1986b). Intraceullar ADP activates K^+ channels that are inhibited by ATP in an insulin-secreting cell line. *FEBS Lett.* **208;** 59-62.

Dunne, M.J. and Petersen, O.H. (1988). Changes in the internal ATP/ADP ratio modulates the gating of K^+ channels in insulin-secreting cells. *Pflügers Arch.* **411;** R111.

Erecinska, M., Bryla, J., Michalik, M., Meglasson, M.D., and Nelson, D. (1992). Energy metabolism in islets of Langerhans. *Biochim. Biophys. Acta* **1101;** 273-295.

Faergeman, N.J. and Knudsen, J. (1997). Role of long-chain fatty acyl-CoA esters in the regulation of metabolism and in cell signalling. *Biochem. J.* **323;** 1-12.

Fan, Z. and Makielski, J.C. (1997). Anionic Phospholipids Activate ATP-sensitive Potassium Channels. *J. Biol. Chem.* **272;** 5388-5395.

Findlay, I. (1987a). ATP-sensitive K^+ channels in rat ventricular myocytes are blocked and inactivated by internal divalent cations. *Pflugers Arch.* **410;** 313-320.

Findlay, I. (1987b). The effects of magnesium upon adenosine triphosphate-sensitive potassium channels in a rat insulin-secreting cell line. *J. Physiol.* **391;** 611-629.

Fraze, E., Donner, C.C., Swislocki, A.L., Chiou, Y.A., Chen, Y.D., and Reaven, G.M. (1985). Ambient plasma free fatty acid concentrations in noninsulin- dependent diabetes mellitus: evidence for insulin resistance. *J. Clin. Endocrin. & Metabol.* **1;** 807-811.

Gilon, P., Shepherd, R.M., and Henquin, J.C. (1993). Oscillations of secretion driven by oscillations of cytoplasmic Ca^{2+} as evidences in single pancreatic islets. *J. Biol. Chem.* **268;** 22265-22268.

Glowarzki, E., Fakler, G., Brandle, U., Rexhausen, U., Zenner, H.P., Ruppersberg, J.P., and Fakler, B. (1995). Subunit-dependent assembly of inward-rectifier K^+ channels. *Proc. R. Soc. Lond. B. Biol. Sci.* **261;** 251-261.

Goode, P.N., Farndon, J.R., Anderson, J., Johnston, I.D.A., and Abascal-Morte, J. (1986). Diazoxide in the Management of Patients with Insulinoma. *World J. Surg.* **10;** 586-592.

Goodner, C.J., Walike, B.C., Koerker, D.J., Ensinck, J.E., Brown, A.C., Chideckel, E.W., Palmer, J., and Kalnasy, L. (1977). Insulin, glucagon and glucose exhibit synchronous sustained oscillations in fasting monkeys. *Science Wash. DC.* **195;** 177-179.

Gribble, F.M., Proks, P., Corkey, B.E., and Ashcroft, F.M. (1998). Mechanism of Cloned ATP-sensitive Potassium Channel Activation by Oleoyl-CoA. *J. Biol. Chem.* **273;** 26383-26387.

Gribble, F.M., Tucker, S.J., and Ashcroft, F.M. (1997). The essential role of the Walker A motifs of SUR1 in K-ATP channel activation by Mg-ADP and diazoxide. *EMBO J.* **16;** 1145-1152.

Gribble, F.M., Tucker, S.J., Seino, S., and Ashcroft, F.M. (1998). Tissue Specificity of Sulfonylureas: Studies on Clonal Cardiac and β-Cell K_{ATP} Channels. *Diabetes* **47;** 1412-1418.

Gross, G.J. and Auchampach, J.A. (1992). Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs. *Circ. Res.* **70;** 223-233.

Grover, G.J., Sleph, P.G., and Dzwonczyk, S. (1992). Role of myocardial ATP-sensitive potassium channels in mediating preconditioning in the dog heart and their possible interaction with adenosine A1-receptors. *Circulation* **86;** 1310-1316.

Grynkiewicz, G., Poenie, M., and Tsien, R.Y. (1985). A new generation of Calcium indicators with greatly improved fluoroscence properties. *J. Biol. Chem.* **260;** 3440-3450.

Gylfe, E., Hellman, B., Sehlin, J., and Taljedal, B. (1984). Interaction of sulfonylurea with the pancreatic B-cell. *Experientia* **40;** 1126-1134.

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* **391;** 85-100.

Hellman, B. (1965). Studies in obese-hyperglycemic mice. *Ann. N.Y. Acad. Sci.* **131;** 541-558.

Henquin, J.C. and Meissner, H.P. (1984). Significance of ionic fluxes and changes in membrane potential for stimulus-secretion coupling in pancreatic B-cells. *Experientia* **40;** 1043-1052.

Hilgemann, D.W. and Ball, R. (1996). Regulation of cardiac Na^+, Ca^{2+} exchange and K_{ATP} potassium channels by PIP_2. *Science* **273;** 956-959.

Horn, R. and Marty, A. (1988). Muscarinic activation of ionic currents measured by a new whole-cell recording method. *J. Gen. Physiol.* **92;** 145-159.

Hu, K., Li, G.-R., and Nattel, S. (1999). Adenosine-induced activation of ATP-senstive K^+ channels in excised membrane patches is mediated by PKC. *Am. J. Physiol.* **276;** H488-H495

Hunter, M. and Giebisch, G. (1988). Calcium-activated K-channels of Amphiuma early distal tubule: inhibition by ATP. *Pflugers Arch.* **412;** 331-333.

Hutton, J.C., Sener, A., Herchuelz, A., Atwater, I., Kawazu, S., Boschero, A.C., Somers, G., Devis, G., and Malaisse, W.J. (1980). Similarities in the stimulus-secretion coupling mechanisms of glucose- and 2-keto acid-induced insulin release. *Endocrinology* **106;** 203-219.

Inagaki, N., Gonoi, T., Clement, J.P.4., Namba, N., Inazawa, J., Gonzalez, G., Aguilar-Bryan, L., Seino, S., and Bryan, J. (1995a). Reconstitution of I_{KATP}: an inward rectifier subunit plus the sulfonylurea receptor. *Science* **270;** 1166-1170.

Inagaki, N., Gonoi, T., Clement, J.P.4, Wang, C.Z., Aguilar-Bryan, L., Bryan, J., and Seino, S. (1996). A family of sulfonylurea receptors determines the pharma-cological properties of ATP-sensitive K^+ channels. *Neuron* **16;** 1011-1017.

Inagaki, N., Tsuura, Y., Namba, N., Masuda, K., Gonoi, T., Horie, M., Seino, Y., Mizuta, M., and Seino, S. (1995b). Cloning and functional characterization of a novel ATP-senistive potassium channel ubiquitously expressed in rat tissues, including pancreatic islets, pituitary, skeletal muscle, and heart. *J. Biol. Chem.* **270;** 5691-5694.

Inoue, I., Nagase, H., Kishi, K., and Higuti, T. (1991). ATP-sensitive K^+ channel in the mitochondrial inner membrane. *Nature* **352;** 244-247.

Kakei, M., Kelly, R.P., Ashcroft, S.J., and Ashcroft, F.M. (1986). The ATP-sensitivity of K^+ channels in rat pancreatic B-cells is modulated by ADP. *FEBS Lett.* **208;** 63-66.

Kurachi, Y., Isomoto, S., Kondo, C., Yamada, M., Matsumoto, S., Higashiguchi, O., Horio, Y., Matsuzawa, Y., and Kurachi (1996). A novel sulfonylurea receptor forms with BIR (Kir6.2) a smooth muscle type ATP-sensitive K^+ channel. *J. Biol. Chem.* **271;** 24321-24324.

Lacy, P.E. and Kostianovsky, M. (1967). Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes* **16;** 35-39.

Lang, D.A., Matthews, D.R., Burnett, M., and Burner, R.C. (1981). Brief, irregular oscillations of basal plasma insulin and glucose concentrations in diabetic man. *Diabetes* **30;** 435-439.

Larsson, O., Ammala, C., Bokvist, K., Fredholm, B., and Rorsman, P. (1993). Stimulation of the K_{ATP} channel by ADP and diazoxide requires nucleotide hydrolysis in mouse pancreatic beta-cells. *J. Physiol.* **463;** 349-365.

Lawson, K. (1996). Potassium channel activation: a potential therapeutic approach. *Pharmacol. Ther.* **70;** 39-63.

Lembert, N. and Idahl, L.-Å. (1998). Alpha-Ketoisocaproate Is Not a True Substrate for ATP Production by Panreatic Beta-Cell Mitochondria. *Diabetes* **47;** 339-344.

Lernmark, A. (1974). The preparation of, and studies on, free cell suspensions from mouse pancreatic islets. *Diabetologia* **10;** 431-438.

Lewis, B., Mancini, M., Mattock, M., Chait, A., and Fraser, T.R. (1972). Plasma triglyceride and fatty acid metabolism in diabetes mellitus. *Eur. J. Clin. Invest.* **2;** 445-453.

Longo, E.A., Tornheim, K., Deeney, J.T., Varnum, B.A., Tillotson, D., Prentki, M., and Corkey, B.E. (1991). Oscillations in cytosolic free Ca^{2+}, oxygen consumption, and insulin secretion in glucose-stimulated rat pancreatic islets. *J. Biol. Chem.* **266;** 9314-9319.

Makhina, E.N. and Nichols, C.G. (1998). Independent Trafficking of K-ATP Channel Subunit to the Plasma Membrane. *J. Biol. Chem.* **273;** 3369-3374.

Malaisse, W.J., Best, L., Kawazu, S., Malaisse-Lagae, F., and Sener, A. (1983). The stimulus-secretion coupling of glucose-induced insulin release: fuel metabolism in islets deprived of exogenous nutrient. *Arch. Biochem. Biophys.* **224;** 102-110.

Malaisse, W.J. and Sener, A. (1987). Glucose-induced changes in cytosolic ATP content in pancreatic islets. *Biochim. Biophys. Acta* **927;** 190-195.

Matthews, D.R., Lang, D.A., Burnett, M.A., and Turner, R.C. (1983). Control of pulsatile insulin secretion in man. *Diabetologia* **24;** 231-237.

Meglasson, M.D., Burch, P.T., Berner, D.K., Najafi, H., and Matschinsky, F.M. (1986). Identification of glucokinase as an alloxan-sensitive glucose sensor of the pancreatic beta-cell. *Diabetes* **35;** 1163-1173.

Meglasson, M.D. and Matschinsky, F.M. (1986). Pancreatic islet glucose metabolism and regulation of insulin secretion. *Diabetes Metab. Rev.* **2;** 163-214.

Neher, E. and Sakmann, B. (1976). Single-channel currents recorded from membrane of denervated frog muscle fibres. *Nature* **260;** 799-802.

Miledi, R., Parker, I., and Sumikawa, K. (1983). Recording of single gamma-amino-butyrate- and acetylcholine-activated receptor channels translated by exogenous mRNA in Xenopus oocytes. *Proc. R. Soc. Lond. B. Biol. Sci.* **218;** 481-484.

Minor, D.L. Jr., Masseling, S.J., Jan, Y.N., and Jan, L.Y. (1999). Transmembrane structure of an inwardly rectifying potassium channel. *Cell* **96;** 879-891.

Nichols, C.G. and Lederer, W.J. (1991). Adenosine triphosphate-sensitive potassium channels in the cardiovascular system. *Am. J. Physiol.* **261;** H1675-H1686

Nichols, C.G., Shyng, S.L., Nestorowicz, A., Glaser, B., Clement, J.P.4., Gonzalez, G., Aguilar-Bryan, L., Permutt, M.A., and Bryan, J. (1996). Adenosine diphosphate as an intracellular regulator of insulin secretion. *Science* **272;** 1785-1787.

Nilsson, T., Arkhammar, P., Hallberg, A., Hellman, B., and Berggren, P.O. (1987). Characterization of the inositol 1,4,5-trisphosphate-induced Ca^{2+} release in pancreatic beta-cells. *Biochem. J.* **248;** 329-336.

Noma, A. (1983). ATP-regulated K^+ channels in cardiac muscle. *Nature* **305;** 147-148.

Panten, U., Biermann, J., and Graen, W. (1981). Recognition of insulin-releasing fuels by pancreatic B-cells: alpha-ketoisocaproic acid is an appropriate model compound to study the role of B-cell metabolism. *Mol. Pharmacol.* **20;** 76-82.

Panten, U. and Christians, J. (1973). Effect of 2-endo-aminonorbornane-2-carboxylic acid upon insulin secretion and fluorescence of reduced pyridine nucleotides of isolated perifused pancreatic islets. *Naunyn-Schmiedebergs Arch. Pharm.*.**276;** 55-62.

Panten, U., Christians, J., Kriegstein, E.v., Poser, W., and Hasselblatt, A. (1974). Studies on the mechanism of L-leucine-and alpha-ketoisocaproic acid-induced insulin release from perifused isolated pancreatic islets. *Diabetologia* **10;** 149-154.

Panten, U., Kriegstein, E.v., Poser, W., Schonborn, J., and Hasselblatt, A. (1972). Effects of L-leucine and α-ketoisocaproic acid upon insulin secretion and metabolism of isolated pancreatic islets. *FEBS Lett.* **20;** 225-228.

Paolisso, G., Gambardella, A., Amato, L., Tortoriello, R., D'Amore, A., Varricchio, M., and D'Onofrio, F. (1995). Opposite effects of short- and long-term fatty acid infusion on insulin secretion in healthy subjects. *Diabetologia* **38;** 1295-1299.

Paucek, P., Yarov-Yarovoy, V., Sun, X., and Garlid, K.D. (1996).Inhibition of the Mitochondrial K_{ATP} Channel by Long-chain Acyl-CoA Esters and Activation by Guanine Nucleotides. *J. Biol. Chem.* **271;** 32084-32088.

Polonsky, K.S., Given, B.D., Hirsch, L.J., llil, H., apiro, E.T., ebe, C., ank, B.H., lloway, J.A., and n Cauter, E. (1988). Abnormal patterns of insulin secretion in non-insulin-dependent diabetes mellitus. *N. Engl. J. Med.* **318;** 1231-1239.

Pralong, W.-F., Bartley, C., and Wollheim, C.B. (1990). Single islet beta-cell stimulation by nutrients: relationship between pyridine nucleotides, cytosolic Ca^{2+} and secretion. *EMBO J.* **9;** 53-60.

Prentiki, M. and Corkey, B.E. (1996). Are the ß-Cell Signaling Molecules Malonyl-CoA and Cytosolic Long-Chain Acyl-CoA Implicated in Multiple Tissue Defects of Obesity and NIDDM. *Diabetes* **45;** 273-283.

Prentki, M., Vischer, S., Glennon, M.C., Regazzi, R., Deeney, J.T., and Corkey, B.E. (1992) Malonyl-CoA and long chain acyl-CoA esters as metabolic coupling factors in nutrient-induced insulin secretion. *J. Biol. Chem.* **267**, 5802-5810.

Quast, U. and Cook, N.S. (1989). Moving together: K^+ channel openers and ATP-sensitive K^+ channels. *Trends Pharmacol. Sci.* **10;** 431-435.

Quayle, J.M. and Standen, N.B. (1994). K_{ATP} channels in vascular smooth muscle. *Cardiovas. Res.* **28;** 797-804.

Rae, J., Cooper, K., Gates, P., and Watsky, M. (1991). Low access resistance perforated patch recordings using amphotericin B. *J. Neurosci. Methods* **37;** 15-26.

Rorsman, P. and Trube, G. (1985). Glucose dependent K^+-channels in pancreatic beta-cells are regulated by intracellular ATP. *Pflugers Arch.* **405;** 305-309.

Sako, Y. and Grill, V.E. (1990). A 48-hour lipid infusion in the rat time-dependently inhibits glucose-induced insulin secretion and B cell oxidation through a process likely coupled to fatty acid oxidation. *Endocrinology* **127;** 1580-1589.

Santos, R.M., Rosario, L.M., Nadal, A., Garcia-Sancho, J., Soria, B., and Valdeolmillos, M. (1991). Widespread synchronous $[Ca^{2+}]_i$ oscillations due to bursting electrical activity in single pancreatic islets. *Pflugers Arch.* **418;** 417-422.

Seino, S. (1999). ATP-Sensitive potassium channels: A model of heteromultimeric Potassium Channel/Receptor Assemblies. *Annu. Rev. Physiol.* **61;** 337-362.

Sener, A., Malaisse-Lagae, F., and Malaisse, W.J. (1981). Stimulation of pancreatic islet metabolism and insulin release by a nonmetabolzable amino acid. *Proc. Natl. Acad. Sci. U.S.A.* **78;** 5460-5464.

Shearman, M.S., Sekiguchi, K., and Nishizuka, Y. (1989). Modulation of ion channel activity: a key function of the protein kinase C enzyme. *Pharmacol. Rev.* **41;** 211-237.

Shyng, S.L., Ferrigni, T., and Nichols, C.G. (1997a). Control of Rectification and Gating of Cloned K_{ATP} Channels by the Kir6.2 Subunit. *J. Gen. Physiol.* **110;** 141-153.

Shyng, S.L., Ferrigni, T., and Nichols, C.G. (1997b). Regulation of K_{ATP} Channel Activity by Diazoxide and MgADP: Distinct Functions of the Two Nucleotide Binding Folds of the Sulfonylurea Receptor. *J. Gen. Physiol.* **110;** 643-654.

Shyng, S.L. and Nichols, C.G. (1997c).Octameric Stoichiometry of the K_{ATP} Channel Complex. *J. Gen. Physiol.* **110;** 655-664

Shyng, S.L. and Nichols, C.G. (1998). Membrane phospholipid control of nucleotide sensitivity of K_{ATP} channels. *Science* **282;** 1138-1141.

Smith, P.A., Ashcroft, F.M., and Rorsman, P. (1990). Simultaneous recordings of glucose dependent electrical activity and ATP-regulated K(+)-currents in isolated mouse pancreatic beta- cells. *FEBS Lett.* **261;** 187-190.

Smith, P.A., Duchen, M.R., and Ashcroft, F.M. (1995). A fluorimetric and amperometric study of calcium and secretion in isolated mouse pancreatic beta-cells. *Pflugers Arch.* **430;** 808-818.

Spruce, A.E., Standen, N.B., and Stanfield, P.R. (1985). Voltage-Depedent ATP-sensitive potassium channels of skeletal muscle membrane. *Nature* **316;** 736-738.

Stagner, J.I., Samols, E., and Weir, G.C. (1980). Sustained oscillations of insulin, glucagon, and somatostatin from the isolated canine pancreas during exposure to a constant glucose concentration. *J. Clin. Invest.* **65;** 939-942.

Standen, N.B., Quayle, J.M., Davies, N.W., Brayden, J.E., Huang, Y., and Nelson, M.T. (1989). Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science* **245;** 177-180.

Sturgess, N.C., Ashford, M.L., Cook, D.L., and Hales, C.N. (1985). The sulphonylurea receptor may be an ATP-sensitive potassium channel. *Lancet* **2;** 474-475.

Stühmer, W. and Parekh, A.B. (1995). Electrophysiological Recordings from *Xenopus* Oocytes. In: *Single-Channel Recording*, 2nd Ed., 341-355. Edited by Sakmann, B. and Neher, E., New York, Plenum Press.

Suzuki, M., Fujikura, K., Inagaki, N., Seino, S., and Takata, K. (1997a). Localization of the ATP-Sensitive K^+ Channel Subunit Kir6.2 in Mouse Pancreas. *Diabetes* **46;** 1440-1444.

Suzuki, M., Kotake, K., Fujikura, K., Inagaki, N., Suzuki, T., Gonoi, T., Seino, S., and Takata, K. (1997b). Kir6.1: A Possible Subunit of ATP-sensitive K^+ channels in Mitochondria. *Biochem. Biophys. Res. Comm.* **241;** 693-697.

Tengholm, A., McClenaghan, N., Grapengiesser, E., Gylfe, E., and Hellman, B. (1992). Glycine transformation of Ca^{2+} oscillations into a sustained increase parallels potentiation of insulin release. *Biochim. Biophys. Acta* **1137;** 243-247.

Terzic, A., Jahangir, A., and Kurachi, Y. (1995). Cardiac ATP-sensitive K+ channels: regulation by intracellular nucleotides and K^+ channel-opening drugs. *Am. J. Physiol.* **269;** C525-C545

Tornheim, K. (1997). Are Metabolic Oscillations Responsible for Normal Oscillatory Insulin Secretion? *Diabetes* **46;** 1375-1380.

Trapp, S., Tucker, S.J., and Ashcroft, F.M. (1997). Actiavtion and inhibition of K-ATP currents by guanine nucleotides is mediated by different channel subunits. *Proc. Natl. Acad. Sci. U.S.A.* **94;** 8872-8877.

Tricarico, D., Barbieri, M., Franchini, C., Tortorella, V., and Camerino, D.C. (1998). Effects of mexiletine on ATP sensitive K^+ channel of rat skeletal muscle fibres: a state dependent mechanism of action. *Br. J. Pharmacol.* **125;** 858-864.

Trube, G., Rorsman, P., and Ohno-Shosaku, T. (1986). Opposite effects of tolbutamide and diazoxide on the ATP- dependent K^+ channel in mouse pancreatic beta-cells. *Pflugers Arch.* **407;** 493-499.

Tucker, S.J., Gribble, F.M., Zhao, C., Trapp, S., and Ashcroft, F.M. (1997). Truncation of Kir6.2 produces ATP-sensitive K^+ channels in the absence of the sulphonylurea receptor. *Nature* **387;** 179-183.

Tusnady, G.E., Bakos, E., Varadi, A., and Sarkadi, B. (1997). Membrane topology distinguishes a subfamily of the ATP-binding cassette (ABC) transporters. *FEBS Lett.* **402;** 1-3.

Vogt, A.M., Ackermann, C., Noe, T., Jensen, D., and Kübler, W. (1998). Simultaneous Detection of High Energy Phosphates and Metabolites of Glycolysis and the Krebs Cycle by HPLC. *Biochem. Biophys. Res. Comm.* **248;** 527-532.

Walker, J.E., Saraste, M., Runswick, M.J., and Gay, N.J. (1982). Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* **1;** 945-951.

Weston, A.H. (1989). Smooth muscle K^+ channel openers; their pharmacology and clinical potential. *Pflugers Arch.* **414 Suppl.;** S99-S105

Williamson, J.R., Corkey, B.E. (1979). Assay of citric acid cycle intermediates and related compounds: update with tissue metabolite levels and intracellular distribution. *Methods Enzymol.* **55;** 200-222.

Wollheim, C.B., Dunne, M.J., Peter-Riesch, B., Bruzzone, R., Pozzan, T., and Petersen, O.H. (1988). Activators of protein kinase C depolarize insulin-secreting cells by closing K^+ channels. *EMBO J.* **7;** 2443-2449.

Xie, L.-H., Takano, M., Kakei, M., Okamura, M., and Noma, A. (1999). Wortmannin, an inhibitor of phosphatidylinositol kinase, blocks the MgATP-dependent recovery of Kir6.2/SUR2A channels. *J. Physiol.* **514;** 655-665.

Yang, J., Jan, Y.N., and Jan, L.Y. (1995). Determination of the subunit stoichiometry of an inwardly rectifying potassium channel. *Neuron* **15;** 1441-1447.

Zaitsev, S.V., Efanov, A.M., Efanova, I.B., Larsson, O., Ostenson, C.G., Gold, G., Berggren, P.O., and Efendic, S. (1996). Imidazoline compounds stimulate insulin release by inhibition of K(ATP) channels and interaction with the exocytotic machinery. *Diabetes* **45;** 1610-1618.

Zhang, J.F., Randall, A.D., Ellinor, P.T., Horne, W.A., Sather, W.A., Tanabe, T., Schwarz, T.L., and Tsien, R.W. (1993). Distinctive pharmacology and kinetics of cloned neuronal Ca^{2+} channels and their possible counterparts in mammalian CNS neurons. *Neuropharmacology* **32;** 1075-1088.

Zhou, Y. and Grill, V.E. (1994). Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle. *J. Clin. Invest.* **93;** 870-876.