Die Rolle und die Regulation der Ionkanäle in den Insel-Zellen

Role and Regulation of Ion Channels in Insulin Secreting Cells

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CONTENTS

ABBREVIATIONS

AmB	Amphotericin B
ANOVA	Analysis of Variance between groups
ASM	Acid sphingomyelinase
ATP	Adenosine tri phosphate
Ca_V channel	Voltage-gated calcium channel
cAMP/PKA	Cyclic adenosine monophosphate/protein kinase A
C2	N-Acetyl-D-Sphingosine
Ca^{2+}	Calcium ion
Cl^-	Chloride ion
DAPI	4′,6′-diamidino-2-phenylindole
DEX	Dexamethasone
Di-C2	Dihydroceramide C2
DMSO	Dimethyl sulfoxide
FCS	Fetal calf serum
g	Gram
GLUT	Glucose transporter
GTP	Guanosine-5'-triphosphate
HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
IAPP	Islet amyloid polypeptide
IGF-1	Insulin-like growth factor-1
K^+	Potassium ion
K_{ATP} channel	ATP-sensitive potassium channel
KCl	Potassium chloride

K_{ir}	Inward rectifier potassium channel
K_v channel	Voltage-gated K+ channel or voltage-dependent K^+ channel
mM	Millimolar (mmol/L)
mV	Millivolt
Na^+	Sodium ion
NaCl	Sodium chloride
PBS	Phosphate buffered saline
PI 3-K	Phosphatidylinositol-3 kinase
PIP2	D-myo-Phosphatidylinositol 3,5-bisphosphate
PKB	Protein kinase B
RT	Room temperature
SEM	Standard error of the mean
SGK	Serum and glucocorticoid inducible protein Kinase
SUR	Sulfonylurea receptor
T2DM	Type 2 diabetes
TEA	Tetraethylammonium chloride
TUNEL	Terminal dUTP nick-end labelling
µM	Micromolar
VDCC	Voltage-dependent calcium channel
VSMC	Vascular smooth muscle cell

Zusammenfassung

Eine große Anzahl von Ionenkanälen und Transportern tragen zur Regulation der elektrischen Aktivität in pankreatischen B-Zellen bei. Die Aktivitäten dieser Kanäle kontrollieren die Ionentransportation, entscheiden sich das Membranpotential und regulieren die Sekretion des Insulins und das Überleben der Zellen.

Die Hormone und die Peptide sind wichtige Regulatoren in den Kanalaktivitäten in den Insel-Zellen. In diesem Studium, demonstrieren wir, dass das Leptin (100 nM), der IGF-1 (50 ng/ml) und das Adrenaline (1 µM) den auswärts Strom anregen und die Zellmembranen hyperpolarizieren. Dadurch hemmt es die Sekretion des Insulins in den Mäuseinsel-Zellen. Die Anwendungen von den Hemmer der PI3-kinase wie LY294002 und Wortmannin kehren die Wirkung der Hyperpolarization um. Es deutet darauf hin, dass die hemmende Wirkungen der Insulinsekretion vom Leptin, IGF-1 und Adrenaline durch PI3-Kinase Signaling Pathway sind. Das exogene Insulin (1 µM) dagegen hat keinen Einfluss an der Kanalaktivität.

Das Amyloid-Peptid ist bekannt in vielen verschiedenen Zellen, die Apoptose zu stimulieren. Die apoptotische Wirkung vermittelt zum Teil durch die Bildung des Ceramids. Das Amyloid beeinflusst besonders mit dem Überleben der pankreatischen B-Zellen. Dieses Studium zeigt, dass das Amyloid und das Ceramid auf die Aktivitäten der Ionkanäle wirkt und das Amyloid auf die Bildung des Ceramids wirkt.

Die Mäuseinsel-Zellen werden isoliert und mit Amyloid $A\beta_{1-42}$ (1-10 µM) oder C2-ceramid (20-60 µM) 1-2 Tagen behandelt. Die Färbung vom TUNEL zeigt, dass das Amyloid und C2 die Apoptose der Insel-Zellen signifikant stimuliert. Die Patch-Clamp-Experimente zufolge werde die Anwendung vom Islet-associated-Polypeptid (IAPP) 5 µM innen 15 Min. den Strom von den K_V-Kanäle der Whole-Zelle deutlich verringert. Die Wirkung des Amyloids werde vom C2 20 µM nachgemacht, hingegen das inactive Di-C2 (20 µM) keine deutlichen Wirkung auf dem auswärts Strom in den Insel-Zellen hat. Laut die Färbung der Immunofluorezenz für das Ceramid, das Amyloid $A\beta_{1-42}$ vermehrt die Bildung des Ceramids. Die Einführung von der Apoptose und der Verringerung des Strom von den K_V-Kanäle bei dem Amyloid werde in acid-Sphingomyelinase-Knockout (ASM-KO) Insel-Zellen aufgehoben. Schließlich, das Amyloid führt die Insel-Zellen zu der Apoptose. Der Effekt ist vermutlich durch die Aktivität von der ASMase, die sich die vermehrten Bildung des Ceramids und die Hemmung der Aktivität der Ionkanäle ergibt.

SUMMARY

A large number of ion channels and transporters contribute to regulation of electrical excitability in pancreatic beta cells. Activities of these ion channels control transportation of ions and determine the membrane potential, eventually regulate insulin secretion and cell survive.

Hormones and peptides are very important regulators in channel activities in insulin secreting cells. In this study we demonstrated, leptin (100 nM), IGF-1(50 ng/ml) and adrenaline (1 µM) induce outward current and hyperpolarization in cell membrane which result in inhibition of insulin secretion in mouse islet cells. Application of PI 3-kinase inhibitors like LY294002 and/or wortmannin reverse the hyperpolarization-induced effect by these hormones. It indicates, the effect of leptin, IGF-1and adrenaline act as inhibitors of insulin secretion through PI 3-kinase signaling pathway. In the contrast, exogenous insulin (1 µM) failed to affect channel activity under the same condition like leptin, IGF-1 and adrenaline.

Amyloid peptides are known to stimulate apoptosis in a wide variety of cells, an effect partially mediated by formation of ceramide. Specifically, amyloid is known to interfere with survival of pancreatic beta cells. As in some cells apoptotic death is paralleled by ceramide dependent alterations of plasma membrane ion channel activity, the present study elucidated the effects of amyloid on ceramide formation and ion channel activity in mouse islets. Mouse islet cells were isolated and treated in cell culture with amyloid peptide $A\beta_{1-42}$ at concentrations between 1 and 10 µM or C2 ceramide (20-60 µM) for 1 – 2 days. As disclosed by TUNEL staining, both, amyloid peptide and C2-ceramide, significantly stimulated apoptotic death of pancreatic islet cells. According to patch clamp experiments, administration of islet associated polypeptide amyloid IAPP (5 µM) within 15 minutes significantly decreased outwardly rectifying whole cell current. The effect of amyloid was mimicked by N-acetyl-D-sphingosine (C2-ceramide, 20 µM), whereas inactive di-ceramide (20 µM) did not significantly modify islet cell outward currents. According to immune fluorescent staining for ceramide, amyloid peptide $A\beta_{1-42}$ increased ceramide formation. Induction of apoptosis and decrease of K_V channel current by amyloid was abolished in islet cells from mice deficient in acid sphingomyelinase. In conclusion, amyloid induces apoptotic islet cell death presumably through activation of acid sphingomyelinase that results in elevated production of ceramide and subsequent inhibition of ion channel activity.

1. INTRODUCTION

1.1 Ion channels and insulin secreting cells

Ion channels are an integral membrane protein or an assembly of several proteins. They facilitate the diffusion of ions across biological membranes through their electrochemical gradient. The conformational change between closed and open state (see Fig. 1.) is called gating.

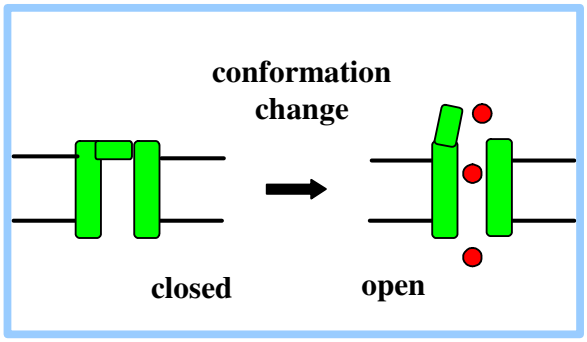


Fig. 1. Channels cycle between open & closed conformations

According to the modulator in gating activity of ion channels, they can be classified to different groups :

- ligand gated channels that are regulated by neurotransmitters

- voltage gated channels that are regulated by the transmembrane potential (electric field)

- second messenger gated channels that are regulated by nucleotides directly or through G-proteins

- mechanosensitive channels that are regulated by osmotic pressure, membrane curvature or sharing forces

- gap junctions or porins are not gated

Ion channels can also be classified according to their ion selectivity :

- K^+ channels
- Na^+ channels
- Ca^{2+} channels
- Cl^- channels
- non-selective cation channels

Ion channels function as electrical signal transducers and they control the electrical properties of all living cells. They also take the responsibility for the regulation of the volume of the cell and participate in intracellular signalling. The physiological process of the nerve impulse is generated by channels mediating the conduction across the synapses, and channels are especially prominent components of the nervous system. In addition, ion channels participate in a wide variety of biological processes that involve rapid changes in cells, such as cardiac, skeletal, and smooth muscle contraction, epithelial transport of nutrients and ions, T-cell activation and pancreatic beta-cell insulin release.

Pancreatic islet cells are endodermally-derived specialized excitable cells that possess many properties of neurons. A large number of ion channels, pumps, and transporters contribute to the regulation of electrical excitability in pancreatic beta-cell. They are responsible for the resting membrane potential (V_m) of the beta cell of about -60 mV when extracellular glucose concentration is lower than 5.6 mM (see Fig. 2.).

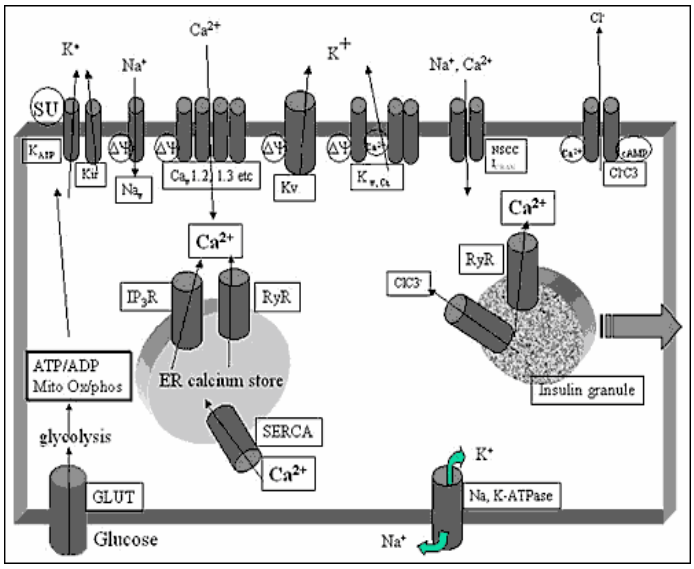


Fig. 2. Beta cell ion channels (from Aschcroft, et al. 1989).

1.2. K_{ATP} channel

In 1968, Dean and Mathews proved for the first time that beta-cells are electrically excitable and that glucose controlled this electrical activity using intracellular micro-electrode recording techniques (Dean,et al.1968; Dean,et al.1970). Later on, the development of patch clamp techniques helped to elucidated the key role of ATP-sensitive potassium (K_{ATP}) channels in the resting membrane potential of the beta-cell as well as the importance of these channels in insulin secretion. Experiments indicated that the initial depolarization induced by glucose is caused by a decrease in the resting membrane permeability to potassium. Thus, the K^+ permeability of the beta cells is a critical determination of glucose-induced insulin release. The experiments have first identified a potassium channel (K^+-channel) that is active at the resting potential and is inhibited by glucose. Closure of this channel requires glucose metabolism (Ashcroft,et al.1984; Cook,et al.1984).

1.2.1 Structure of K_{ATP} channel

These potassium channels belong to the inward rectifier (K_{ir}) subfamily. The name of K_{ir} came from the fact that these channels conduct K^+ current into the cell more easily than to the outside of the cell. Using Kir3.1 cDNA as a probe, Kir6.1 as a novel Kir member was identified from a rat islet cDNA library (Cook,et al.1984). Northern blot analysis showed that Kir6.2 mRNA is expressed at very high levels in pancreatic islets and in insulin secreting cells MIN6 and HIT-cells and is expressed relative lower in other organs which are known to have K_{ATP} channels, like skeletal muscle, heart and brain. However, expression of mouse Kir6.2 alone in COS-1 cells, HEK239 cells or Xenopus leavis oocytes did not induce any significant current, indicating that Kir6.2 alone does not function as a K_{ATP} channel (Inagaki,et al.1995).

Functional K_{ATP} channels are only formed when Kir6·2 is expressed together with sulfonylurea receptor 1 (SUR1) . The SUR1 is a regulatory protein channel that was finally cloned after numerous experiments using the partial peptide sequences only in 1995 (Inagaki,et al.1995). Sulfonylurea, however, such as Tolbutamide, glyburide are used in the treatment of type 2 diabetes since more than 50 years. This 1582 amino acid protein SUR1 is expressed at high levels in pancreatic islets and pancreatic beta cell lines including MIN6, RINm5F and HIT (Inagaki et al.1995). The SUR1 is a member of the ATP binding cassette (ABC) family of

membrane proteins, which includes the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel among others (Higgins.1992; Aguilar-Bryan et al.1999). The Kir6.2-SUR1 complex forms the functional K_{ATP} channel. The complete channel consists of a core of four Kir 6.2 subunits surrounded by four SUR1 subunits (Inagaki et al.1995; Sakura et al.1995). The SUR1 complex acts as a regulator of the K^+ channel, binding ATP as well as sulfonylurea compounds. Both ATP and tolbutamide have inhibitory actions on the K_{ATP} channel and therefore inhibit K^+ efflux (see Fig. 3). Each K_{ir} channel subunit folds into the membrane to form two transmembrane domains (M_1 and M_2) surrounding a pore loop (P). The transectional model shows Kir6.2 subunits surrounded by their accompanying SUR1 subunit (see Fig. 4.).

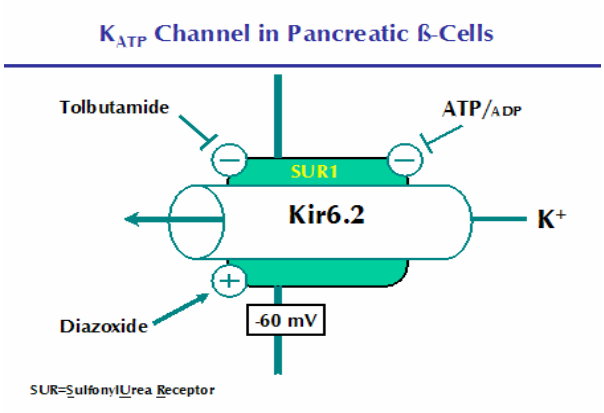


Fig.3. The Kir6.2-SUR1 complex (from Miki, et al. 1999).

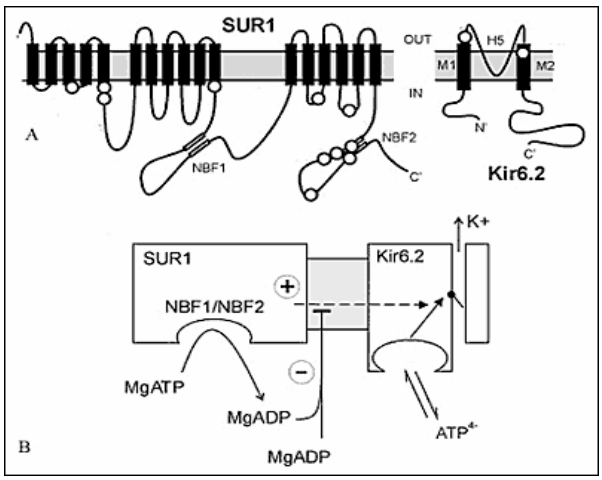


Fig.4. Transection of K_{ir} channels: The structure reveals that K_{ir} channels consist of four subunits: each subunit folds into the membrane to form two transmembrane domains (M_1 and M_2) surrounding a pore loop. The four pore loops line the central ion-conducting pore with the M_1 and M_2 subunits (from Miki, et al. 1999).

The isoforms of SUR have been cloned, SUR1, SUR2A and SUR2B. SUR2A, a divergent from of SUR2 but highly homologous to SUR1 was found in 1996 (Isomoto et al. 1996) . This subunit is a component of the smooth muscle K_{ATP} channel, which the heart K_{ATP} channel is composed of Kir6·1 and SUR2B. The tissue and cells distribution of SUR1, SUR2A, Kir6·1 and Kir6·2 assessed by Northern blot analysis is summarized in Table 1(Chutkow et al.1996; Yamada et al.1997).

Table 1. Tissue distribution of K_{ATP} channel subunits. The levels of expression represent the relative abundance of mRNA of each subunit among different tissues (from Miki, et al. 1999).

	SUR1	SUR2A	Kir6·1	Kir6·2
Pancreatic islets	+++	++	++	+++
MIN6	+++	−	−	+++
HIT	+++	−	−	+++
RINm5F	±	−	−	+
Brain	+	++	++	++
Heart	−	+++	+++	++
Skeletal muscle	±	+++	++	++

− undetectable; ± very low levels; + low levels; ++ moderate levels; +++ high levels.

1.2.2 Function of K_{ATP} channel

K_{ATP} channels play a regulatory role in many cellular functions such as hormone secretion, excitability of neurons and muscles and cytoprotection in heart and brain ischemia, by coupling the cell metabolism to electrical activity (Ashcroft.1998c; Terzic et al.1995). Since the primary function of insulin secreting cell is secretion of insulin, the K_{ATP} channels in pancreatic beta-cells are critical in the regulation of glucose-induced and sulfonylurea-induced insulin secretion. That glucose-induced insulin secretion is dependent on the closure of the K_{ATP} channels has in

principle become accepted (Cook et al.1998).

Elevation of plasma glucose concentration to more than 8 mM results in the depolarization of the beta cell. Glucose is taken up into the beta-cell by the glucose transporter GLUT2 and is metabolized through glycolytic pathways and oxidized in the mitochondria yielding in the generation of ATP. An increase in the ATP/ADP ratio causes closure of the K_{ATP} channels and depolarization of the cell via the decreased K^+ permeability (Rorsman et al.1985) (see Fig.5). Under resting conditions, K_{ATP} channels are open due to the interaction of ADP with SUR1.

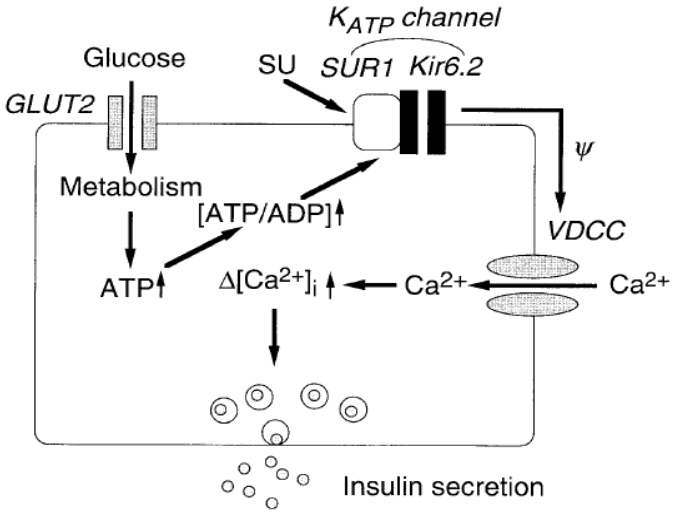


Fig. 5. K_{ATP} channel plays a crucial role in glucose-induced and sulfonylurea-induced insulin secretion.(GLUT2, glucose transporter 2; VDCC, voltage-dependent calcium channel; SU, sulfonylurea; Ψ, membrane depolarization.) (from Miki, et al. 1999)

Mutations in either the K_{ir} or SUR1 can result in K_{ATP} channel defects. It has been found that Persistent Hyperinsulinemic Hypoglycemia of Infancy (PHHI), also referred to as familial hyperinsulinism or pancreatic nesidioblastosis, a rare disease is caused by mutations of SUR1 and K_{ir}6.2 that result in constitutionally closed channels (Huopio.2002). This disease is characterized by inappropriate high levels of insulin secretion despite severe hypoglycemia.

1.2.3 The regulation of the K_{ATP} channel

Several ligands affect K_{ATP} channel activity. Both ATP (with or without Mg^{2+}) and tolbutamide have inhibitory effects on the K_{ATP} channel and inhibit K^+ efflux. This leads to depolarization of the pancreatic beta-cell, stimulation of Ca^{2+} influx and insulin secretion. ADP and pharmacological K^+ channel openers, such as diazoxide, stimulate the K_{ATP} channels and promote K^+ efflux, that result in membrane repolarization and inhibition of insulin secretion (see Fig. 6).

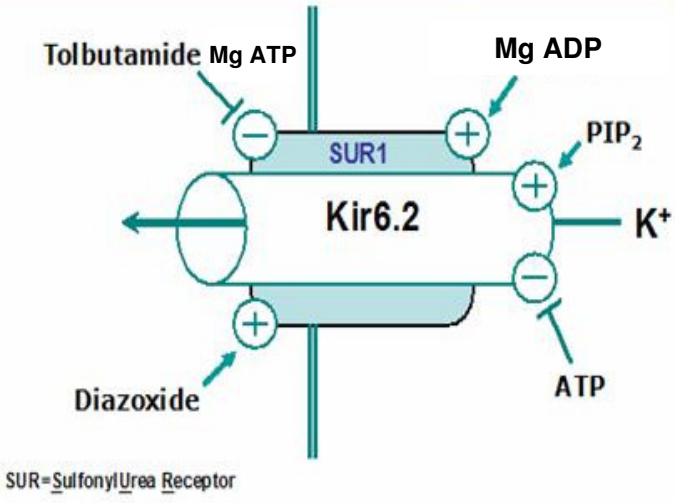


Fig. 6. Regulators of K_{ATP} channel: ATP inhibits, and PIP_2 activates, by direct interaction with Kir6.2 subunits. Sulphonylureas inhibit, and diazoxide activates, by interaction with the SUR subunit. In addition, in the presence of Mg^{2+} and ADP can activate the channel through interaction with the NBFs of SUR.

1.2.3.1 ATP and ADP

It is well accepted, K_{ATP} channels are controlled by intracellular ATP and ADP concentrations or the ATP/ADP ratio (Ashcroft et al.1990; Seino et al.2000). ATP inhibits K_{ATP}

channels, while ADP opens them. Single channel recording of K_{ATP} channel activity using the patch clamp method showed that channel open in an ATP-free solution and close by brief application of MgATP at millimolar concentrations (Cook.1984; Noma.1983; Trube et al.1984).

Although Kir6.2 contains no obvious consensus sequences for nucleotide binding, a single mutation in this subunit (K185Q) dramatically reduces the ability of ATP to inhibit channel activity (Tucker et al.1997). This observation demonstrats that ATP inhibits K_{ATP} channels by binding to Kir6.2 .

Application of non-hydrolysable analogues of ATP, and of ATP in the absence of Mg^{2+} still resulted in inhibition of channel activity (Ashcroft et al.1989b). It suggests that ATP hydrolysis is not necessary for channel inhibition and that binding of the molecule is sufficient to induce K_{ATP} channel closure.

The comparison of ATP, GTP and ITP revealed that only ATP causes high affinity channel inhibition (Tucker et al.1998) . It suggests that elements unique to the adenine ring are critical for the specificity of nucleotide binding and/or for the ability of nucleotide binding to induce the channel closure. The ability of ATP and ADP, but not AMP, to inhibit the channel suggests that more than the a-phosphate of the nucleotide is required for the interaction (Tucker et al.1998).

1.2.3.2 Sulfonylurea compounds

K_{ATP} channels are targets of anti-hyperglycemic compounds. The best-known drugs that regulate K_{ATP} channels are the sulfonylureas. Sulfonylureas are widely used to treat type 2 diabetes mellitus in humans, acting as insulin secretagogues by binding to SUR1, blocking pancreatic beta-cell K_{ATP} channel and triggering insulin release (Proks et al.2002).

Distinct sulfonylureas have different binding affinities for SUR subtypes. Some of them are very specific for SUR1 (e.g. nateglinide, mitiglinide), some are moderately specific for SUR1 (e.g. glyburide, glimepiride), and others are essentially not selective (e.g. repaglinide) (Quast et al.2004).

The mechanism of Sulfonylurea by which they induce the closure of K_{ATP} channels is best explained by a two-site model. In the absence of nucleotide, the dose-response for tolbutamide to block K_{ATP} currents shows both a high-affinity (Ki = 2.0 microM) and a low-affinity (Ki = 1.8 mM) binding site (Gribble et al.1997). It has also been demonstrated that the high-affinity site resides on SUR1, whereas the low-affinity site is located on Kir6.2. Because the concentrations

required to inhibit the Kir6.2 subunit are much higher than those found in the plasma of patients treated with the drugs, it is suggested that the low-affinity site has no clinical relevance (Ribalet et al.1996). Binding of sulfonylureas to the cytoplasmic domains of SUR1 ultimately results in closure of the pore formed by Kir6.2. There must be a close physical association between the sulfonylurea binding site on SUR and the Kir6.2 subunit (Aguilar-Bryan et al.1999). The exact transduction process is still unclear.

1.2.3.3 Diazoxide and other channel openers

A diverse group of compounds, referred to as potassium channel openers (KCOs) are able to open K_{ATP} channels and thus hyperpolarize the cells. Diazoxide, pinacidil, nicorandil, cromakalim and RP-49356 belong to this group of drugs.

It has been shown that inhibition of the K_{ATP}-mediated current by an initial application of 100 mM ATP was partly reversed by the addition of 100 mM diazoxide (Ashcroft et al.1989a and b). The target binding site of potassium channel openers is proposed to be the sulfonylurea receptor, based on observations that the response of reconstituted K_{ATP} channels to either diazoxide, cromakalim, or pinacidil correlates with the SUR subtype (Inagaki et al.1996; Isomoto et al.1996). Diazoxide does not competitively displace glibenclamide at the K_{ATP} channel, suggesting that it has a other binding site on the channel than the sulfonylureas (Panten et al.1989).

The study about KCOs demonstrated that treatment with KCO compounds can indeed lead to inhibition of insulin secretion and metabolic "rest" of beta-cells. Eventually KCOs preserve the beta-cell function by reducting insulin secretion in a rat diabetes model (Kresten et al.2004).

Except being used for the treatment of hyperinsulinemic states or as smooth muscle relaxants reducing hypertension, channel openers also have been tested in asthma and cardiovascular disease. Intracerebroventricular administration of K^+ channel "openers" block experimentally induced seizures in rodents through the hyperpolarization of neurons. K^+ channel openers may also be useful in the treatment of neurodegenerative diseases, pain and cerebral ischemia (Gehlert et al.1994).

1.2.3.4 Phosphatidylinositol bisphosphate (PIP_2)

Phosphatidylinositol phosphates have been found to affect ion channel activity. Exposure of K_{ATP} channels to 5 µM Phosphatidylinositol bisphosphate (PIP_2) reduces the inhibitory effect of ATP and blocked the activation of channel by diazoxide. Prolonged application of 10 µM PIP_2 completely removes inhibition of K_{ATP} channels by 1 mM ATP (Baukrowitz et al.1998). Similar to PIP_2, phosphatidylinositol-4-phosphate (PIP) is also able to reduce the inhibitory effect of ATP. Whereas phosphatidylinositol (PI) has no obvious effect on K_{ATP} channels even at a 10 times higher concentration (Baukrowitz et al.1998). PIP_2 also reduces the maximal block produced by saturating concentrations of tolbutamide (Koster et al.1999) and glibenclamide (Krauter et al.2001).

The mechanism by which PIP_2 and PIP open K_{ATP} channels involves an action on the $K_{ir}6.2$ subunit and a shift in ATP sensitivity. PIP_2 incorporates into the cell membrane and interacts with the carboxyl terminus of the K_{ATP} channel. This interaction distorts the ATP-binding site and prevents ATP from binding. In the absence of PIP_2 the channel is able to bind ATP, that causes the channel to close (Frances et al.1998). PIP_2 therefore stabilizes a state in which the channel can not interact with ATP (Alexey et al.1998).

1.2.3.5 Regulation by other factors

Experiments without nucleotides ATP/ADP indicate that guanosine-5'-triphosphate (GTP) (10 microM to 1 mM) and guanosine diphosphate (GDP) (100 microM to 1 mM) evoke dose-dependent K_{ATP} channels activation in RINm5F-cells (Dunne et al.1986; Findlay.1987).

Cations and anions also influence the open probability of the K_{ATP} channel. It has been shown that 5 mM free Mg^{2+} is required to produce 50% inhibition of K_{ATP} channel activity in rat pancreatic beta-cells and millimolar concentrations of divalent cations produce an inhibition in channel activity (Ashcrof et al.1989b). Other experiments suggest that the open probability of the channel is increased 2- to 10-fold when $C1^-$ is substituted by glutamate or aspartate (Tabcharani et al.1989). The effects of cytoplasmic pH on the K_{ATP} channel appear to interact with those of ATP (Misler et al.1989). In the presence of ATP, channel activity increases when the intracellular

pH (pH_i) is enhanced to 7.9 and decreases when pH is reduced to 6.3 in inside-out patches from rat beta-cells. However, changes of pH_i in ATP-free solution have little effect on channel activity (Misler et al.1989).

At physiological concentrations, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) inhibit Kir6.2/SUR1 currents through the interaction with the nucleotide inhibitory site of Kir6.2. This inhibition is dependent on the presence of SUR1, which may act by increasing the width of the nucleotide-binding pocket of Kir6.2 (Dabrowski et al.2003). They may therefore contribute to the resting level of channel inhibition in the intact cell.

1.3 Voltage-dependent K^+ (K_v) channel

Voltage-dependent or voltage-gated K^+ channels (K_v channels) are present in all animal cells. They open and close upon changes in the transmembrane potential. K_v channels are one of the key components in generation and propagation of electrical impulses in the nervous system and heart. In the pancreas during the physiological process of insulin secretion, increased glucose metabolism depolarises beta-cells by closing K_{ATP} channels. This triggers action potential due to the opening of voltage-dependent Ca^{2+} channels. Repolarisation of pancreatic beta-cell is mediated by the activation of K_v channels.

1.3.1 Structure of K_v channel in beta cell

The tetrameric structure of K_v channels is made of two functionally and structurally independent domains: an ion conduction pore and voltage-sensor domains. The ion conduction pore is made of four α-subunits which are arranged symmetrically around the conduction pathway. Voltage-sensor domains are positioned at the periphery of the channel and consist of four transmembrane segments (S1-S4) (Vanessa et al.2003) (see Fig. 7). The pore-forming α-subunits and modulatory β-subunits exist in the membrane as one component of macromolecular complexes, able to integrate a myriad of cellular signals that regulate ion channel behavior (Torres et al.2007). In response to the membrane potential, the voltage-sensor domains

structurally rearrange, particularly S4, which includes positively charged amino acids at every third position, leads to conformational changes in the conduction pore, which opens or blocks the ion conduction pathway (Fatemeh et al.2006).

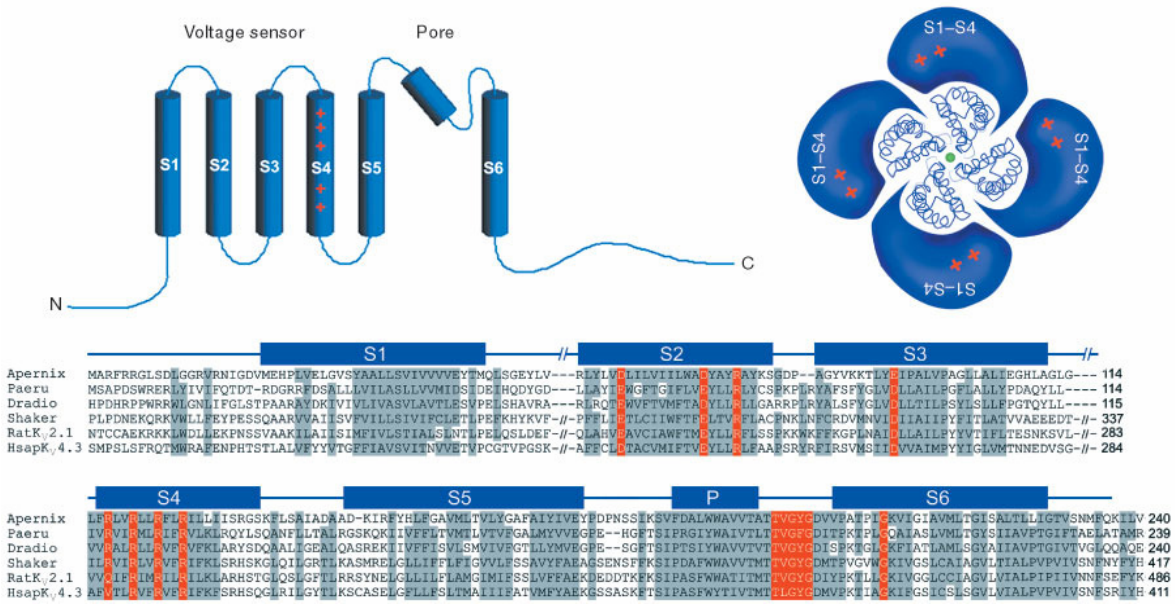


Fig. 7. Architecture of a voltage-dependent K^+ channel: Transmembrane-spanning segments (S1–S6) are labelled (dark segments); S1–S4 form the voltage sensor and S5–S6 form the pore. Four subunits surround the pore (Ruta, et al. 2003).

Numerous studies have been carried out to detect subunits of K_v channels (MacDonald et al.2001and2002; Ling et al.2004). Using reverse transcriptase-polymerase chain reaction (RT-PCR) identification of mRNA transcripts and using Western blot and immuno-histochemical studies to detect relevant subunits expressed abundantly at the protein level, a large number of K_v channel subunits have been detected in insulin secreting cells (MacDonald et al.2003b).

The first report of K_v2.1 expression in an insulin-secreting cell (βTC-neo) was detected by RT-PCR method (Roe et al.1996a). Later afterwards using a dominant-negative strategy, MacDonald and his colleagues demonstrated that K_v2.1 contributes 60 to 70% of the voltage-

dependent outward K^+ current in rat beta-cells and HIT-T15 insulinoma cells . They also proved that K_v channels regulate glucose-induced insulin secretion in rat islets (MacDonald et al.2001). The experiments established that $K_v2.1$ mediates the majority of the voltage-dependent outward K^+ current in rat and mouse beta-cells (MacDonald et al.2001; Tamarina et al.2005).

K_v currents resistant to tetraethylammonium (TEA) but blockable by 4-aminopyridine (4-AP) were recorded from mouse pancreatic islet cells. These channels are opened at voltages more positive than -40 mV, display a rapid turn on and inactivate completely within 250 ms (Findlay et al. 1985; Smith et al.1989). Expression of $K_v1.4$, $K_v1.5$, $K_v2.1$, $K_v2.2$, $K_v3.1$ and $K_v3.2$ were detected in INS-1 cells whereas other Kv channels, $K_v1.1$, $K_v1.2$, $K_v1.3$, $K_v1.6$ and $K_v3.4$ were not detected (Su et al. 2001). So far, there are at least 11 mammalian Kv channel families and various related families (EAG related, KCNQ or KvLQT and KCa) (see Table 2) (Yasuda et al. 2008; Ullrich et al. 2005; MacDonald et al.2003b; Sano et al.2002; Christie MJ.1995; Dilks et al.1999; Hugnot et al.1996; Ottschytsch et al.2002; Salinas et al.1997; Stocker et al.1998; Bauer et al.2001; Robbins.2001). Among these channel families, members of the Kv1, Kv2, and Kv3 channel families regulate currents have been observed in pancreatic beta-cells (Dukes et al.1996; Xu et al.1995; Blaine et al. 1998; Koch et al. 1997).

Tab.2. Family members and current types of mammalian Kv channel (from MacDonald et al.2003b).

	Kv1 (shaker)	Kv2 (shab)	Kv3 (shal)	Kv4 (shaw)	Modulatory α-subunits	Kv Related	K_{Ca}
Family Members	1.1 1.2 1.3 1.4 1.5 1.6 1.7	2.1 2.2	3.1 3.2 3.3 3.4	4.1 4.2 4.3	5.1 6.1, 6.2, 6.3 7.1 8.1 9.1, 9.2, 9.3 10.1 11.1	EAG related: *EAG(1, 2)* *ERG(1–3)* *ELK(s, e, 2)* KCNQ(1–5)	BK1 (slo1) BK2 (slo2) BK3 (slo3) SK1 SK2 SK3 SK4
Current Type	delayed-rectifier (1.1, 1.2, 1.3, 1.5, 1.7) and A-current (1.4)	delayed-rectifier	delayed-rectifier (3.1, 3.2) and A-current (3.3, 3.4)	A-current	do not form functional channels alone but can interact with and modulate some other channels (notably Kv2.1)	delayed-rectifier (EAG, ELKs/e); voltage-dependent inward rectifier (ERG, ELK2); slow delayed-rectifier (KCNQ)	Ca^{2+}-sensitive delayed-rectifier; large (BK) and small (SK) conductance; SK shows little voltage-dependence

EAG—ether-a-go-go; ERG—eag-related gene; ELK—eag-like K^+channel

1.3.2 Function of K_v channel in insulin-secreting cells

Many K_v channel genes, represented by more than 40 mammalian isoforms (termed K_v1 to K_v9) (Coetzee et al.1999), give rise to overlapping functions, primarily regulating repolarization of the plasma membrane. Experiments involving inhibition of K_v channels have shown that K_v channels play an important role in regulating beta-cell calcium oscillations in response to glucose stimulation. In pancreatic beta cells, K_v channels are potential mediators of repolarization, closure of Ca^{2+} channels, and limitation of insulin secretion (Ullrich et al. 2005b).

In rat islets and insulinoma cells, inhibition of $K_v1.4$, 1.6, and 2.1 channels with tetraethylammonium (TEA) enhances glucose-induced insulin secretion by 2- to 4-fold (MacDonald et al.2001; Su et al. 2001; Ullrich et al. 2007).

Studies in 1977-1980 have observed that the general antagonist of K_v and Ca^{2+}-activated K(KCa) channel TEA prolongs mouse beta-cell action potentials. These are the earliest studies showing that repolarising outward K^+ currents modify insulin secretion and that inhibition of the channels enhances insulin secretion from rat islets (Atwater et al.1979; Henquin.1977; Henquin et al.1979; Herchuelz et al.1980). Later afterwards, numerous experiments have demonstrated the stimulatory effect of TEA on insulin secretion (MacDonald et al.2001; Henquin.1990; Philipson et al.1994; Eberhardson et al.1996; Roe et al.1996a; Gopel et al.1999). Mutations in K_v and related channels are known to play important roles in disorders such as familial long Q-T syndrome, episodic ataxia type 1, benign familial neonatal convulsion, familial and thyrotoxic hypokalemic periodic paralysis, and autosomal dominant deafness (Hatta et al.2000). Genes encoding both $K_v1.7$ and $K_v3.3$ have been mapped to a region of chromosome 19 (19q13.3–13.4) containing a diabetes susceptibility locus (Kalman et al.1998). Polymorphism of K_v channels or their numerous regulatory proteins could lead to loss or gain of function. Increased K_v channel function would shorten glucose-stimulated insulin secretion by causing premature repolarisation of the action potential and decreased K_v channel function could lead to beta cell over-excitability and increased cytosolic Ca^{2+}, possibly leading to apoptosis through activation of the caspase cascade and induce of oligonucleosomal DNA fragmentation (Chandra et al.2001). Other experiments show that $K_v1.3$ -/- mice have an increased basal metabolic rate and are resistant to the development of obesity in response to a high fat diet (Xu et al.2003).

1.3.3 Modulation of K_v channel

1.3.3.1 Syntaxin

Research in brain synaptosomes has shown the physical and functional interactions between syntaxin 1A and K_v channels. K_v channels may play a role in synaptic efficacy and neuronal excitability. Particularly, syntaxin 1A was found to associate with K_v1.1 and Syntaxin affected the channel amplitude. At low syntaxin concentrations there was a significant increase in amplitudes, and at higher concentrations, however, the amplitudes decreased, probably because of a concomitant decrease in cell-surface channel expression (Fili et al.2001). Additionally, it has been demonstrated that the activity of the delayed-rectifier K(+) channels (K(DR)) channel subtype, K_v1.1, identified by its specific blocker dendrodotoxin-K, is inhibited by SNAP-25 (synaptosome-associated protein of 25 kDa) in insulinoma HIT-T15 beta cells (Ji et al.2002). A co-precipitation study of rat brain confirmed that SNAP-25 interacts with the K_v1.1 protein. It also has been reported that SNAP-25 blocked K_v outward currents from rat beta-cells by approximately 40% (MacDonald et al.2002d). But when K_v2.1 channels had been functionally knocked out using a dominant-negative approach, SNAP-25 had no effect on outward currents in beta cells anymore. It was determined that the inhibitory effect of SNAP-25 was specific to K_v2.1 in primary rat beta cells as compared to other K_v channels. It also has been shown that the C-terminus of the channel associates to syntaxin. The study shows that overexpression of syntaxin 1A disrupts membrane targeting of cloned K_v2.1 through an interaction with the C-terminus and reduces K_v outward currents in rat beta cells (Leung et al.2003).

1.3.3.2 Cyclic adenosine monophosphate/protein kinase A (cAMP/PKA)

The cyclic adenosine monophosphate/protein kinase A (cAMP/PKA)-signalling pathway has been implicated in the regulation of K_v channels (Choquet et al.1987). Elevation of intracellular cAMP modulates K_v channels, induces a rapid decrease in the peak potassium outward current in murine lymphocytes (Choquet et al.1987), GH4C1 pituitary cell line (Chung et al.1995), and a human melanoma cell line (Nilius et al.1992). But the opposite result was observed in cardiac myocytes (Walsh et al.1988). An increase in K_v channel outward current was induced by epinephrine which can induce inhibition of the glucose-dependent increase in $[Ca^{2+}]i$ in ob/ob and wild-type mouse beta cells (Fournier et al. 1993) as the effect is reversed by TEA.

Isoproterenol also increases the ultrarapid delayed rectifier K^+ current (Ikur, appears to correspond to K_v1.5 cloned channels) in human atrial cells in a concentration-dependent manner (Li et al.1996). It seems that hormonal modulation of K_v currents is physiologically important for diverse cell function. Application of 8-Br-cAMP to K_v 2.1 expressed in Xenopus oocytes causes a voltage-independent elevation of current amplitude, whereas application of PKA leads to a reduction in K_v2.1 current (Wilson et al.1994).

1.3.3.3 Glucagon-like peptide-1 (GLP-1)

Beta cell K_v channels are the targets of the G-protein coupled glucagon-like peptide-1 (GLP-1) receptor, which takes part in glucose metabolism and is physiologically linked to the modulation of insulin secretion (MacDonald et al.2002a). MacDonald and his colleagues have found that GLP-1 and the GLP-1 receptor agonist exendin-4 inhibit K_v outward currents by approximately 40% in rat beta cells using patch clamp in the whole-cell configuration. This prolongs the time-course of beta-cell repolarisation (MacDonald et al.2002b). Additionally, it has been shown that the mechanism may involve activation of phosphatidylinositol-3-kinase (PI3-K) gamma (Li et al.2006).

1.3.3.4 NADPH and others

In rat pulmonary artery smooth muscle cells thromboxane A(2) (TXA(2)) analog U46619 inhibits K_v currents, an effect that is strongly prevented by the NADPH oxidase inhibitor apocynin (Cogolludo et al.2006). It suggests activation of NADPH oxidase and the subsequent production of hydrogen peroxide are involved in the Kv channel inhibition. The study from MacDonald et al (MacDonald et al.2003a) demonstrates that intracellular NADPH/NADP+ ratio can increase the intracellular redox potential. This causes beta-cell K_v2.1 currents to inactivate quickly and completely. This may be important since the metabolic generation of NADPH could reduce the efficacy of K_v channels in repolarising the beta-cell. Research group of Ullrich has demonstrated, that activity of Kv1.5 can ben upregulated through SGK1 stimulated by dexamethasone (Ullrich et al. 2005b).

1.4 Voltage-gated calcium (Ca_V) channel

Voltage-gated calcium (Ca_V) channels are ubiquitously expressed in humans and animals. They are found in a variety of cells including excitable cells and some nonexcitable cells (Yang et al. 2005b).

1.4.1 Structure and function

Ca_V channels have been divided into three families, Ca_{V1}, Ca_{V2}, and Ca_{V3}, consisting of closely related members (Ertel et al.2000). Ca_V channels are almost all composed of the $Ca_V\alpha1$, $Ca_V\beta$, and $Ca_V\gamma$ subunits; some also contain the $Ca_V\alpha2$ δ subunit (Takahashi et al.1987; Glossmann et al.1987; Hosey et al.1987; Leung et al.1987) (see Fig. 8.). In this complex, only the Ca $_V\alpha1$ subunit is large enough to contain four homologous transmembrane domains forming a Ca2+ conducting pore (Takahashi et al.1987). Other subunits, $Ca_V\beta$, $Ca_V\gamma$ and $Ca_V\alpha2$ δ subunits, are referred to as auxiliary subunits.

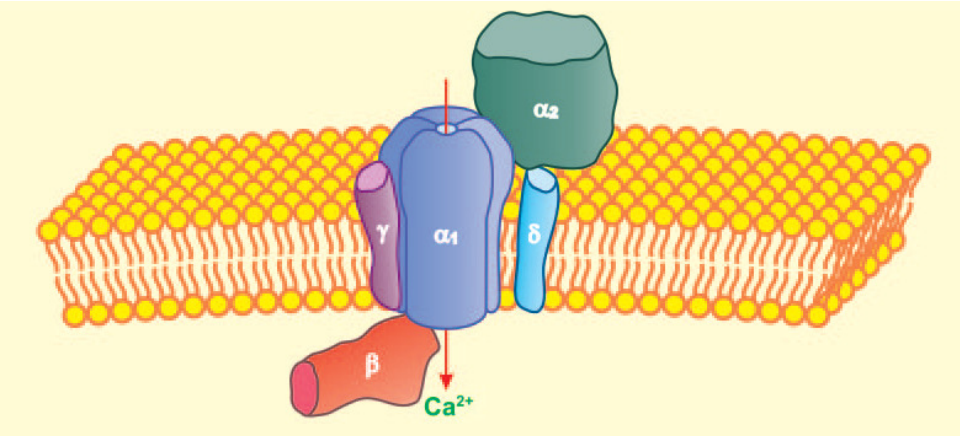


Fig. 8. A structural model of Ca_V channels: Ca_V channel subunit assembly in the plasma membrane. Pore-forming subunits $Ca_V\alpha1$ complex with auxiliary subunits $Ca_V\beta$, $Ca_V\gamma$ and $Ca_V\alpha2$ δ to form functional Ca_V channels in the plasma membrane (from Yang et al.2006).

The conformational change of Ca_V channels begins from an impermeable state to a highly

permeable pore is triggered by membrane depolarization. The highly permeable pore enables the rapid influx of extracellular Ca^{2+} into the cytosol where Ca^{2+} serves as a second messenger coupling electrical signaling to Ca^{2+}-dependent protein-protein interactions and enzymatic reactions (Catterall et al.2000).

Ca_V channels mediate Ca^{2+} influx in various cell types throughout the body. It controls a diverse range of cellular processes including exocytosis, endocytosis, muscle contraction, synaptic transmission, and metabolism (Catterall et al.2000; Berridge et al.1998). Ca_V channels play an important role in beta cell physiology and pathophysiology as they take part in the process of glucose-mediated insulin secretion (Yang et al.2005b). Point mutation in the human $Ca_V1.2$ gene results in excessive insulin secretion (Splawski et al.2004). Trinucleotide expansion in the human $Ca_V1.3$ and $Ca_V2.1$ gene is revealed in a subgroup of patients with type 2 diabetes.

It has been shown that the increase of beta cell Ca_V channel activity and/or of channel density results in enhanced insulin exocytosis and more efficient regulation of glucose homeostasis (Yang et al.2005a). On the other hand, the decrease of Ca_V channel activity and/or of channel density causes inhibition of insulin secretion and glucose intolerance. Reduced Ca_V channel activity has been associated with type 2 diabetic patients (Roe et al.1996b; Iwashima et al.1993; Sharp.1996). Beside the control of insulin secretion, beta cell Ca_V channels are also involved in beta cell development, survival, and growth (Namkung et al.2001; Jing et al.2005; Sjoholm.1995; Popiela et al.1991).

Glucose metabolism in the beta cell leads to an increase in ATP production resulting in closure of K_{ATP} channels, depolarization of the plasma membrane, opening of Ca_V channels, and thereby release of insulin. Beta cell Ca_V channels devote themselves to maintain a satisfactory concentration of Ca^{2+}. Inappropriate regulation of beta cell Ca_V channels causes beta cell dysfunction and even apoptosis as demonstrated in both type 1 and type 2 diabetes (Roe et al.1996b; Namkung et al.2001; Jing et al.2005).

1.4.2 Regulation of Cav channel

PI3K is involved in Ca_V channel modulation. Expression of PI3Kγ massively enhances current density without altering activation and inactivation kinetics of $Ca_V1.2/\beta2\alpha$ channels expressed in COS-7 cells (Viard et al.2004).

Activation of PKA increases Ca_V1 channel activity in mouse pancreatic beta-cells as

demonstrated by the use of the perforated-patch whole-cell configuration (Kanno et al.1998; Gillis et al.1993; Ammala et al.1993).

In the mouse pancreatic beta cell, acute application of a PKC activator does not affect Ca_V channel activity. However, Ca^{2+} influx through beta-cell Ca_V channels dramatically decreases after deprivation of PKC (Arkhammar et al.1994).

Inhibition of Serine/threonine protein phosphatases (PPs) with okadaic acid can slightly increases Ca_V channel activity in mouse pancreatic beta-cells. However, after activation of PKA with forskolin, the activity of Ca_v channel was significantly enhanced by okadaic acid (Sim et al.2003).

The regulation of pancreatic beta-cell Ca_V channels by G protein-coupled receptors has been also reported (Ammala et al.1992; Isaev et al.2004; Scholze et al.2001). Activation of glucagon receptors has been found to regulate Ca_V channels in the insulin-secreting cell line HIT cells and an increase in $[Ca^{2+}]i$ in response to glucagon was demonstrated (Rajan et al.1989) . Instead of glucagon receptors, the glucagon-like peptide-1 (GLP-1) receptor has become a pharmaceutical target in the treatment of type 2 diabetes. The activity of GLP-1 receptor induces glucose-induced insulin release at least in part through enhancement of beta-cell Ca_V channel activity. The effect is characterized by an increase in Ca_V current amplitude and a positive shift in voltage-dependent inactivation (Salapatek et al. 1999).

Elevated FFAs (free fatty acids) can also affect beta-cell Ca_V channel activity. Extracellular administration of palmitate at a concentration of 1 mM on top of 15 mM glucose further increases $[Ca^{2+}]i$ in mouse islets. Simultaneously, patch clamp measurements show increases in whole-cell Ca_V currents and cell capacitance by extracellular administration of palmitate (Olofsson et al.2004).

1.5 Na^+ channels

Voltage dependent Na^+ currents can be identified in pancreatic beta-cell by their rapid activation and inactivation during a maintained depolarization and by the sensitivity to the Na^+-channel blocker tetrodotoxin (TTX) (Plant.1988; Rorsma et al.1986; Satin et al.1988; Pressel et al.1990; Hiniart et al.1988). Glucose-stimulated electrical activity in canine beta-cells is abolished by TTX indicating a significant role for Na^+-currents in beta-cell electrical activity, and

thus possibly insulin release, in this species (Pressel et al.1990).

1.6 Cl^- channels

Kinard and Satin reported the first time that insulin-secreting cells have a Cl^- channel current (Kinard et al.1995). The current is activated by hypotonic conditions, glyburide and intracellular cAMP. They proved that the channel current is mediated by Cl^- channels, since replacing $[Cl^-]o$ with less permeant aspartate reduces current amplitude and depolarizes its reversal potential. Cl^- channel activation would be expected to depolarize islet membrane potential. This anion-permeable pancreatic islet beta cell Cl^- channel is an outwardly rectifying Cl^- channel which mediates a large inward current. The channel is activated by cell swelling (induced by the application of hypotonic solution) or by a rise in intracellular cAMP (Kinard et al.1995).

1.7 Ion channel activities and apoptosis in beta cell

Apoptosis is reported for the first time by Kerr et al in 1972 (Kerr et al.1972). This term is often called "programmed cell death" as it is an innate response of the cell which protects the rest of the organism from a potentially harmful agent and this leads to a variety of morphological changes, including blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, formation of “apoptotic bodies” and chromosomal DNA fragmentation (Kerr et al.1965; Kerr et al.1972; Wyllie et al.1980; McConkey et al.1998). In contrast to apoptosis, necrosis is characterized by a dramatic increase in cell volume and rupture of the plasma membrane (Gores et al.1990). This release of the dying cells contents into the extracellular space can cause further tissue damage by affecting neighboring cells or by attracting proinflammatory cells (Haslett et al.1992) (see Fig. 10.).

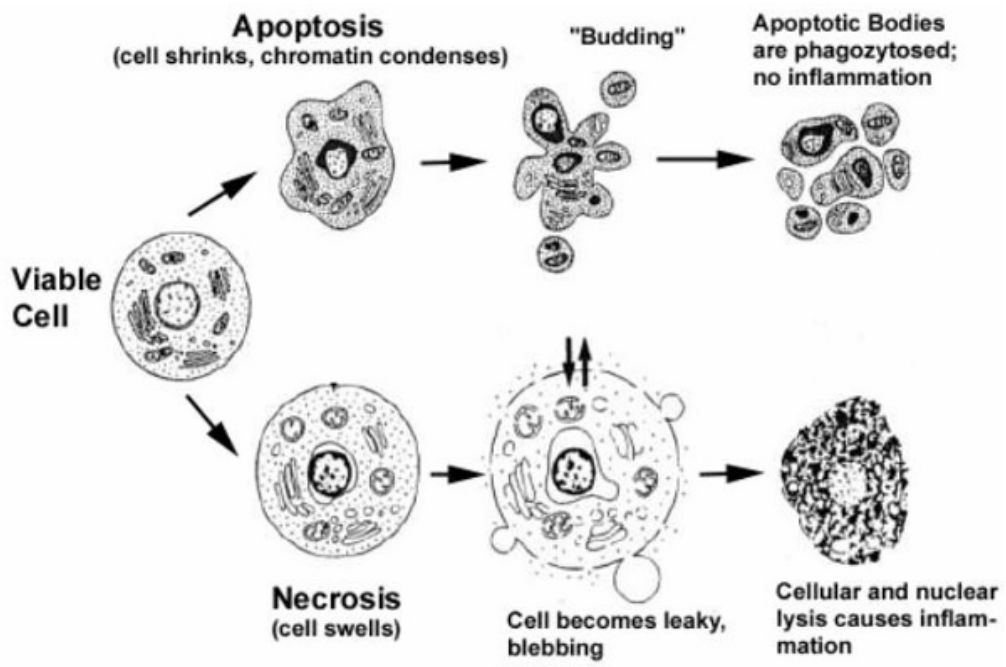


Fig.10. Hallmarks of the apoptotic and necrotic cell death process. Apoptosis includes cellular shrinking, chromatin condensation and margination at the nuclear periphery with the eventual formation of membrane-bound apoptotic bodies that contain organelles, cytosol and nuclear fragments and are phagocytosed without triggering inflammatory processes. The necrotic cell swells, becomes leaky and finally is disrupted and releases its contents into the surrounding tissue resulting in inflammation (Modified from Van Cruchten et al. 2002).

Apoptosis is one of the main types of programmed cell death that occurs during several pathological situations in multicellular organisms and builds up the mechanism of cell replacement, tissue remodeling, and removal of damaged cells. In both type 1 and type 2 diabetes, beta cell death is thought to occur by apoptosis. The mechanism have been to be triggered by specific proteases(so called caspases) (Specifically, activation of a family of intracellular cysteine proteases) (Alnemri et al.1996). Several mediators are involved in the activation of apoptosis, including death receptor activation, oxidative stress (McConkey et al.1994) and Ca^{2+}.

Animal models of diabetes suport that Fas/FasL system, perforin, TNF, or interleukin (IL)-1β as effectors of apoptotic islet cell death (Chervonsky et al.1997; Itoh et al.1997; Kagi et

al.1997; Kagi et al.1999).

Ca^{2+} influx and subsequent Ca^{2+}-dependent apoptotic signals are involved in beta-cell death (Choi et al.2007; Iwakura et al.2000). Activation of L-type Ca^{2+}-channels is associated with DNA fragmentation and induces apoptosis and specific blockers of these channels prevent the activation of endonuclease was prevented (Juntti-Berggren et al.1993). Sustained high concentrations of glucose cause increases in the cytosolic Ca^{2+} and oligonucleosomal DNA fragmentation (Efanova et al.1998). Effect of high glucose–induced DNA fragmentation was inhibited by diazoxide, an opener of K_{ATP} channels that hyperpolarizes beta-cell membranes. Diazoxide treatment indeed has been found to elevate Bcl-2/Bax ratio accordance to ameliorate the insulin secretory capacity and action (Huang et al.2007). Chronic exposure to Sulfonylureas (glibenclamide, tolbutamide and nateglinide) accelerates apoptotic beta-cell death (Takahashi et al.2007).

The major function of beta-cells is to synthesize and secrete of insulin. Apart from insulin, beta-cells also produce amylin (Moore et al.1991), known as islet amyloid polypeptide (IAPP). Amylin functions as part of the endocrine pancreas and contributes to glycemic control.

IAPP polymerizes upon section and forms insoluble extracellular plaques. It deposits up to 70-90% of patients with type 2 diabetes (T2DM) (Clark et al.1990; Clark et al.1996). More and more evidences suggest that abnormal aggregation of IAPP has a role in beta-cell death in T2DM, but the mechanism remains unclear. Recent study suggests that amyloid induces an increase in $K(_V)3.4$ and caused an increase in I(A) current amplitude carried by $K(_V)3.4$ channel subunits in NGF-differentiated PC-12 cells and hippocampal neurons (Pannaccione et al.2007). In human neuroblastoma cell line (SK-N-SH cells), L-type calcium channel blocker nifedipine or decreased extracellular Ca^{2+} concentration blocked amyloid-beta induced cell death (Neurochem. 2004). In another research in beta-cells found reverse results: neither the Ca^{2+} channel blocker verapamil nor a cytosolic Ca^{2+} buffer protect beta-cells from human amyloid(hA)-evoked apoptosis (Bai et al. 1999). These results suggest that alterations in cytosolic Ca^{2+} homoeostasis do not contribute to the pathway of apoptosis evoked by hA in beta-cells. The role of ion channel activity and apoptosis induced by amyloid in beta-cells remains elusive.

2. AIM OF THE STUDY

Membrane ion channels regulate the biological function of beta-cells. While pathological changes in ion channel activities link to T2DM.

This study was undertaken to elucidate possible mechanisms that inhibit insulin secretin and may be involved of apoptotic cell death. For this purpose, the patch clamp method was used to examine ion channel activities in isolated mouse islet cells. As glucose-induced changes of membrane potential depend on intact cellular metabolism. The perforated patch clamp configuration was used to examine membrane potential changes. Effects on ion channels and whole cell current were examined with the standard whole cell configuration.

In the first part the role of PI3K in the regulation of membrane potential was analysed by the use of pharmacological inhibitors and with reduced expression of 80% 3'-phosphoinositide-dependent kinase-1 hypomorphic mice ($PDK1^{hm}$).

The second set of experiments elucidates effects of amyloid peptides on ion currents in insulin secreting cells. A special focus of this study was to examine the role of ceramide by the use of the cells isolated from the acid sphingomyelinase kock-out (ASM-KO) mice.

3. MATERIALS AND METHODS

3.1 Cell preparation

3.1.1 Mouse islets isolation

In this study, mice of the strain C57BL6/NCrl, phosphoinositide-dependent kinase-1 hypomorphic mice (*pdk1*^{hm}), *pdk1*^{wt}, acid sphingomyelinase knock-out (ASM-KO)ASM (-/-) and wild type littermates ASM (+/+) mice aged from 3-6 months were used in all experiments. They were killed by inhalation of CO_2 . After mouse desinfection, a long cross incision was cut on the abdomen, exposing the opening of the general bile duct in the surface of the duodenum. The right and left sides of the opening were knotted and then the termination of the bile duct with the gallbladder was knotted as near to the liver as possible. A small incision on the opening was cut out and used for injection of 3 ml collagenase solution (see Tab. 4). After successful injection and swelling of the pancreas, it was cut out from other adjacent organs and tissues. Collagenase digestion was performed for 10 minutes at 37°C in a water bath. The procedure was terminated by addition of 30 ml ice-cold Hank's solution (see Tab. 3). Thereafter the pancreatic fragments were sedimented by centrifugation (150 g for 1 minute at RT) and the supernatant removed. The tissue pellet was again resuspended in Hank's solution up to 30 ml. After 1 minute centrifugation, the pellet was resuspended in 3-6 ml ice-cold Hank's solution and sieved through a tea sieve in a clean Ø 9 cm pertri dish with a black bottom. White islets were isolated from exocrine tissue under a dissection microscope and collected by hand selection. In avarage 126.19 ± 6.48 islets were isolated from a mouse (n = 207 preparations).

Tab. 3. Ingredients of isolating solutions of mouse islet cells

Compositions:

Hank's stock Solution(4×)1L			Hank's stock Solution(1×)1L	
Compound	Unit (g)	Concentration (mM)	Compositions	Concentration (mM)
NaCl	32	547.5	NaCl	136.88
KCl	1.6	21.46	KCl	5.36
$CaCl_2 \times 2H_2O$	0.74	5.03	$CaCl_2 \times 2H_2O$	1.26
$MgSO_4 \times 7H_2O$	0.8	3.25	$MgSO_4 \times 7H_2O$	0.81
$Na_2HPO_4 \times 2H_2O$	0.24	1.35	$Na_2HPO_4 \times 2H_2O$	0.34
KH_2PO_4	0.24	1.76	KH_2PO_4	0.44
$NaHCO_3$	1.4	16.67	$NaHCO_3$	4.17
HEPES	9.5	39.86	HEPES	10
PH=7.25			PH=7.25	

Preparation:

Hank's solution			Collagenase solution	
Compositions	volume	Concentration (mM)	Compositions	volume
Hank' stock sol.	100 ml		Without BSA Hank' sol.	13 ml
H_2O	300 ml		1M $CaCl_2$	70 µl
Glucose	0.22 g	3.05	Collagenase	14.1 mg
BSA	1.16 g			
PH=7.25				

3.1.2 Primary beta cells preparation and culture

After collection of islets, sterile 10 ml ice-cold PBS (Ca^{2+}/Mg^{2+}-free, pH=7.2, Gibco- cat#. 20012-068) was added. After centrifugation (150 g for 1 minute at RT), PBS was removed and 300-400 µl trypsin solution (1/4 dilution with Ca^{2+}/Mg^{2+}-free PBS of 0.5 g/L of trypsin) was added. The islets were then kept for 4 minutes at 37°C. The digestion was stopped by addition of 10 ml culture medium. After centrifugation (300 g for 4 minutes at RT), the supernatant was

removed and culture medium added to the cell pellet. After 4 minutes of centrifugation, the supernatant was removed and all the pellet resuspended in a small volume of new culture medium (200 µl/100 islets). 20-25 µl of the suspension were dropped in the middle of the petri dishes for patch clamp experiment. Cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS) (v/v), 10 mM Hepes, 1 mM Na-pyruvate, 2 mM L-glutamine, 50 i.u. penicillin/ml and 50 µg streptomycin/ml (see Tab. 4). For fluorescence microscopy cells were seeded on glass cover slips coated with poly-L-ornithine. The dishes were placed about 60-120 minutes in the incubator in order to get the cells attached on cover slip or dish bottom. The dishes were then filled with 2 ml culture medium. About 8-10 dished were prepared from 100 islets (Ullrich et al.2005). Cells were kept in incubator 1-3 days according to different experiments and were treated as indicated in each experiment.

Tab. 4: Ingredients of islet cell culture medium

Cell culture medium	
Total volume	ml
RPMI 1640	450
HEPES 1M	5
Pyruvate (100mM)	5
Penicillin (5000 i.u./ml)/Streptomycin(5000µg/ml)	5
FCS	50
L-Glutamin (200mM)	5

3.2 Chemicals and reagents

Adrenaline	Sigma-Aldrich, Deisenhofen, Germany
Alexa fluor 488 anti-mouse IgG	Invitrogen, Paisley, UK

4-Aminopyridine	Sigma-Aldrich, Deisenhofen, Germany
Amphotericin B from Streptomyces	Sigma-Aldrich, Deisenhofen, Germany
Amyloid β-protein fragment 1-42	Sigma-Aldrich, Deisenhofen, Germany
Amyloid β-protein fragment 42-1	Bachem
Collagenase NB8	Serva, Heidelberg, Germany
Dexamethasone	Sigma-Aldrich, Deisenhofen, Germany
4′,6′-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich, Deisenhofen, Germany
Diabetes associated peptide amide human (IAPP)	Sigma-Aldrich, Deisenhofen, Germany
Dihydroceramide C2	Sigma-Aldrich, Deisenhofen, Germany
Dimethylsulfoxide (DMSO)	Sigma-Aldrich, Deisenhofen, Germany
D-myo-Phosphatidylinositol3,5-bisphosphate (PIP2)	Echelon, Salt Lake City, U.S.A
In Situ cell death detection kit Fluorescein	Roche, Mannheim, Germany
Insulin from bovine pancreas	Sigma-Aldrich, Deisenhofen, Germany
Insulin-like growth factor-1 (IGF-1)	Sigma-Aldrich, Deisenhofen, Germany
Leptin from mouse	Sigma-Aldrich, Deisenhofen, Germany
LY-294002 hydrochloride	Sigma-Aldrich, Deisenhofen, Germany
Monoclonal Antibody to Ceramide(MID 15B4)	Alexis, Lörrach, Germany
N-Acetyl-D-Sphingosine (C2)	Sigma-Aldrich, Deisenhofen, Germany
Ouabain	Sigma-Aldrich, Deisenhofen, Germany
Poly-L-ornithine	Sigma-Aldrich, Deisenhofen, Germany
Rapamycine	Sigma-Aldrich, Deisenhofen, Germany
RU-486	Sigma-Aldrich, Deisenhofen, Germany
Tetraethylammonium chloride (TEA)	Sigma-Aldrich, Deisenhofen, Germany
Tolbutamide	Sigma-Aldrich, Deisenhofen, Germany
Wortmannin	Sigma-Aldrich, Deisenhofen, Germany

Leptin, insulin and IGF-1 were prepared as stock solutions in normal saline and further diluted in bath solution containing 0.1%(w/v) bovine serum albumin as a carrier. C2, Di-C2, dexamethasone, wortmannin and LY294002 were stored as stock solutions in DMSO.

3.3 PATCH CLAMP EXPERIMENTS

The patch clamp technique allows to measure currents through cell membranes using the voltage clamp mode of the technique. Membrane potential can be measured in the current clamp mode.

3.3.1 Voltage Clamp

The voltage clamp technique allows to measure current that flows through the membrane at any particular potential hold at a constant value. The principle of the technique is to inject a current which is equal in amplitude but opposite in sign to the current flowing across the cell. This results in no net current flow across the membrane, and the membrane potential remains constant. Therefore, by measuring the current, which has to be injected to clamp the potential, the current flowing across the membrane can be determined. By measuring currents at different clamped membrane potentials it is then possible to characterize voltage-dependency ion currents.

3.3.2 Patch Clamp

The use of the voltage clamp technique was further extended by the invention of the patch clamp technique almost thirty years ago by Neher and Sakmann. Neher and Sakmann wanted to record from a tiny area (the so-called patch) of a surface membrane by pressing a fire-polished pipette against a living cell. In 1976 they reported the first single-channel current recording of an acetylcholine-activated channel (Neher et al.1976). This technique allows direct electrical

measurements of ion channel currents while simultaneously controlling the cellular membrane potential. In 1981 they showed that a clean glass pipette can fuse to cell membrane to form a seal of an unexpected high resistance and mechanical stability (Hamill et al.1981) . This seal was called gigaseal since it can have an electrical resistance as high as 10^9.

The figure below is a conceptual diagram of the patch-clamp setup.

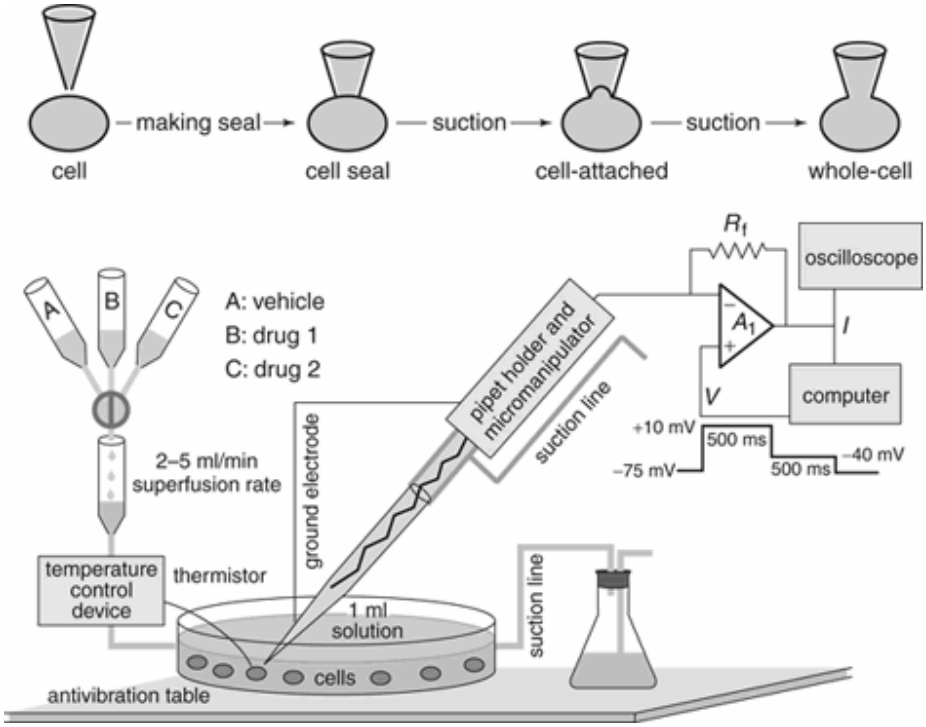


Fig.11. Conceptual diagram of the patch-clamp setup (from Crumb WJ et al. 2003).

A fine-tipped glass capillary is used to make contact with a biological cell membrane to form a high resistance seal. Conventionally, this requires the use of a microscope, and a micromanipulating device connected to the capillary. A sensing electrode inside the capillary is connected to a high-gain, trans-impedance amplifier circuit that converts the current flowing

through the electrode to an output voltage. The purpose of the high-resistance seal is to insure that the flow of current from the sensing electrode inside the capillary to the ground electrode in the bath solution is predominantly due to ions flowing through the cell membrane.

One of the advantages of the patch clamp technique is that it allows measurements on very small cells such as mammalian cells. The basic principle of this technique is that a fluid filled micropipette containing a silver/silver chloride electrode is brought in contact with the cell membrane. By applying gentle suction to the pipette, a giga-seal is formed between the pipette and the cell membrane.

This giga-seal permitts four distinct recording configurations: The cell-attached patch mode, the inside-out patch mode, the outside-out patch mode and the whole-cell patch mode (see Fig.12). When the pieptte is sealed to the cell membrane, single channels can be recorded in the on-cell or cell-attached patch mode.

After formation of the giga-seal, further suction can rupture the patch of membrane under the tip of the micropipette, providing access for the microelectrode to the interior of the cell; this situation is termed the whole cell configuration. The whole cell configuration allows to measure the sum of all of the ion channels activities in the cell membrane (the whole-cell current or macroscopic current). The standard whole cell configuration allows manipulation of the intracellular solution, but has the concomitant disadvantage that soluble cytosolic constituents are lost from the cell by dialysis with the pipette solution. The perforated patch whole-cell configuration avoids this problem.

To prevent the disadvantage of "wash-out" (for instance, by loss of second messengers, changes in enzyme activity or modification of the phosphorylation state) by conventional whole-cell configuation, the perforated patch (also called permeabilized patch) was developed (Horn et al.1988) . The perforation of the patch of membrane under the pipette is produced by pore-forming compounds added to the pipette solution. Most of them are polyenes, such as nystatin and amphotericin B (AmB) are often used (see Fig. 13).

In whole-cell configuration, the part of the membrane inside the capillary is disrupted, thereby creating a low-resistance pathway between the sensing electrode and the cytoplasm. From an electrical viewpoint, this puts the sensing electrode in the glass capillary inside the cell. This allows for the control of the cell membrane via the non-inverting input of the trans-impedance amplifier. The current measurement between the sensing electrode and the bath

electrode is then the resultant sum of all of the ion currents in the remainder of the cell, which, due to the small geometry of the capillary, comprises the majority of the ion channel currents in the "whole cell" (Hille.1992; Hamill et al.1981).

Furthermore, by retracting the micropipette in either the cell-attached or the whole-cell configuration, a small patch of membrane can be isolated in either inside-out or out-side out configuration. These two configurations give the opportunity to measure single channel currents under circumstances facilitating pharmacological manipulations from both the cytoplasmic and extracellular side of the membrane.

One disadvantage of current implementations of the patch-clamp technique is the high level of manual intervention required to make the measurements, which severely restricts the measurement throughput. Typically, an experienced scientist can patch and make measurements from 10-20 individual cells per day. This level of measurement throughput is far below that required for secondary (hundreds to thousands / day) or primary (thousands to tens of thousands / day) pharmaceutical drug screening and has limited the ability to screen ion channel targets with high fidelity.

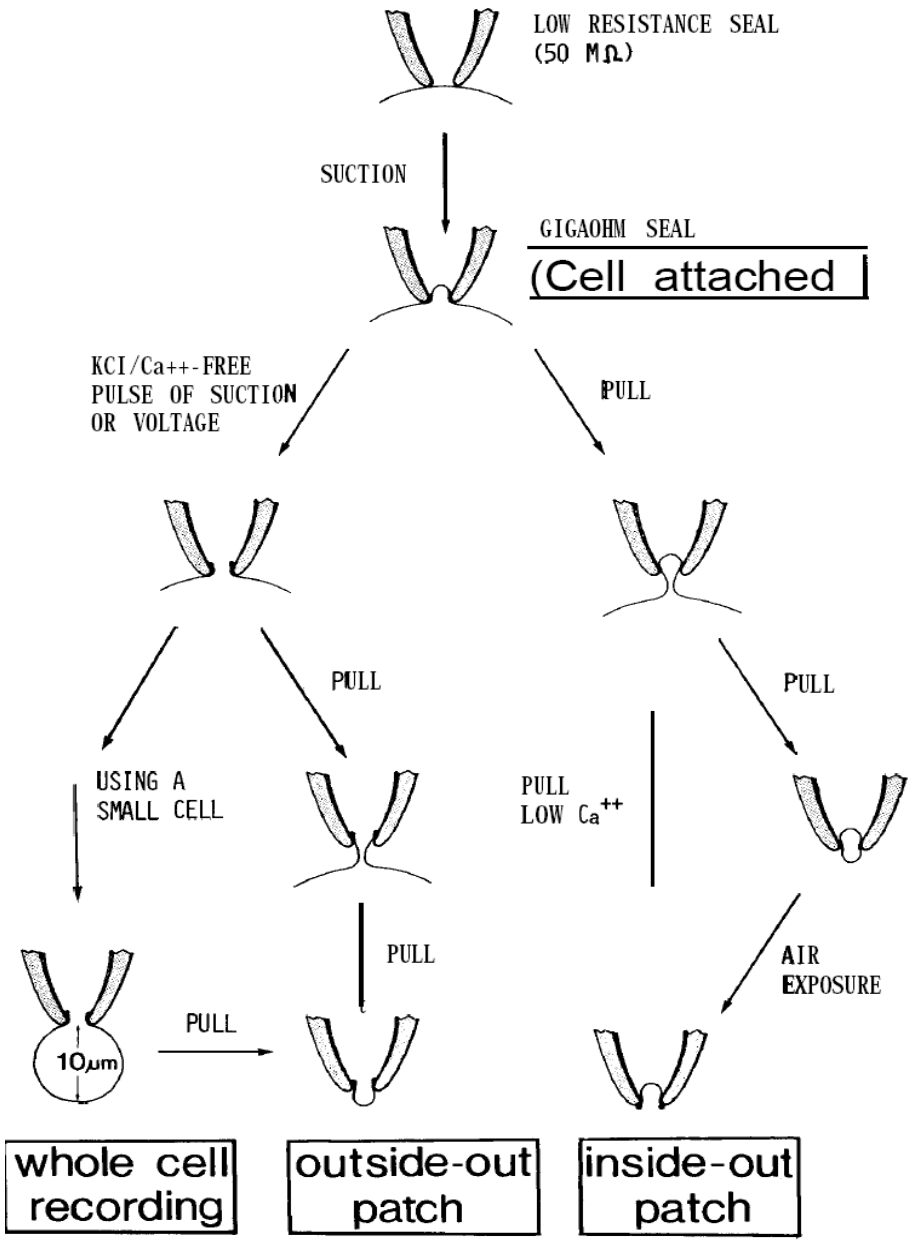


Fig.12: Schematic representation of the procedures that lead to the different patch clamp configurations. The on-cell configuration is obtained by forming a giga-seal between the patch pipette and the cell membrane. Withdrawal of the pipette from the cell produces an inside-out patch. If the patch is destroyed in the on-cell configuration, the standard whole-cell configuration is obtained. Withdrawal of the pipette from the standard whole-cell configuration produces an outside-out patch (from Hamill et al. 1981).

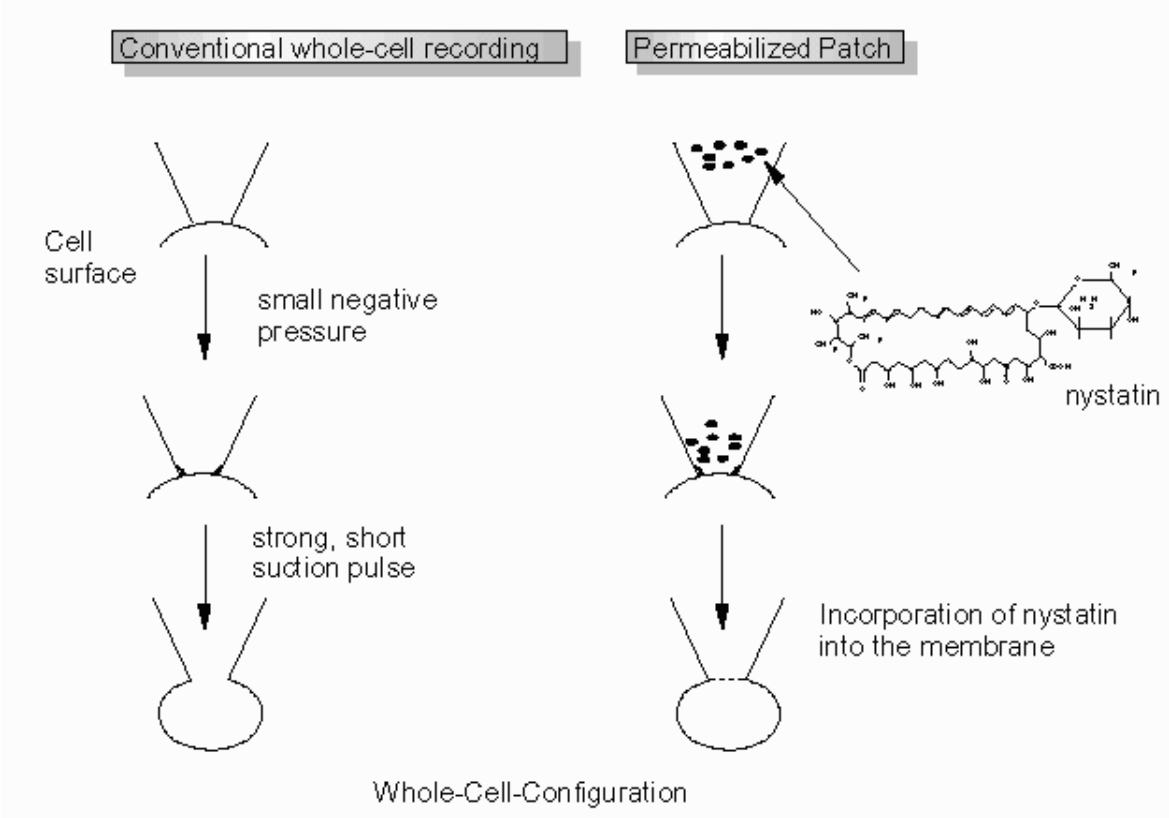


Fig.13: Schematic representation of the procedures that lead to the conventional whole-cell configuration and permeabilized whole-cell configuration. Perforated patch whole-cell configuration is produced if the patch is permeabilised instead of destroyed (from Hamill et al. 1998).

3.3.3 Patch Clamp Experiment

3.3.3.1 Pipette

Pipettes can be made from many different type of glass. Glass capillaries are available from soft or hard glasses. Hard glass pipettes often have a narrow shank after pulling and consequently a higher resistance. Hard glasses tend to have better noise and relaxation properties. Borosilicate glass is one kind of hard glass and has low dielectric loss and is desirable for the lowest-noise

recordings. During the experiment borosilicate glass capillaries (GC150TF-7.5, 1.5mm O.D.×1.17mm I.D. Harvard Apparatus LTD, UK) were used. The pipettes were pulled by DMZ universal puller (Zeitz, Augsburg, Germany) from borosilicate micropipettes and heat-polished. Pipettes are pulled in two stages: the first to thin the glass to 200-400 µm at the narrowest point over a 7-10 mm region, and the second to pull the two halves apart, leaving clean , symmetrical breaks. Both halves can be used after heat polish.

3.3.3.2 Solutions

Tab. 5: Ingredients of solutions during patch clamp experiments

Bath solution	
compositions	mmol/l
NaCl	145
KCl	5
$MgCl_2.6H_2O$	1
$CaCl_2$	2.5
Hepes	10
Adjust with NaOH	PH 7.4
Glucose	5.6
Pipette solutions	
For K channel exp.:	
compositions	mmol/l
K-Gluconat	95
KCl	30
$MgCl_2 \cdot 6\ H_2O$	1
EGTA	3
$CaCl_2$	1

NaH_2PO4	1.2
Na_2HPO4	4.8

For perforated patch measurements:

Amphotericin B 1 mg/ml was prepared before use and was protected from light.

3.3.3.3 Experimental procedures

The culture dish with attached primary mouse islet cells was mounted onto the stage of an inverted microscope. The volume of the recording chamber was 0.2-0.3 ml. Solutions were superfused through the chamber at a rate of 5-6 ml/min. The experiments were carried out at room temperature (20°C). Patch clamp experiments were performed in the whole-cell configuration by the use of an EPC9 patch-clamp amplifier (HEKA Electronic Company, Germany). Pipettes had a resistance of 4.0-6.0 Ω when filled with the pipette solution .

When the patch-pipette was approached to the cell, the resistance increased to 10.0-16.0 Ω. After applying a slow and gentle suction to the pipette, a tight seal (2-20 GΩ) was established in normal bath solution, the cell-attached configuration was formed. After a strong and sudden suction, the patch of cell membrane broke and a conventional whole-cell configuration was formed. For perforated patch configurations, a pipette solution containing amphotericin B was used. After formation of a cell-attached configuration, perforated whole-cell configuration was formed slowly after 5-10 minutes.

Only stable current and voltage measurements were used for analysis, i.e. when the access resistance was stable and lower than 20 MΩ.

3.3.3.4 Recording and analysis

Experiments were performed using whole-cell current clamp recording to monitor membrane potential and using whole-cell voltage clamp mode to examine membrane currents. During voltage clamp recordings, the membrane potential was held at -70 mV, and 20 mV steps of 400 ms duration were applied every 1 s (range -100 mV to 80 mV). Current and voltage were measured using an EPC9 amplifier (Heka Elektronik, Germany), and currents evoked in response

to the voltage step protocol were analyzed using Pulse+Pulse Fit V8.53 software (Heka Elektronik, Germany).

3.4 Measurement of cell death

For fluorescent staining, primary beta cells were plated onto poly L-ornithine-coated glass cover slip. Cells obtained from approximate 100 islets were distributed onto 6 glass cover slips and were seeded at a density of 1-1.2x10^4 cells/dish.

3.4.1 Poly-L-ornithine coating

In order to improve the attachment of the cells to glass, the glass cover slips (22 x 22 mm) were coated with 0.01% L-ornithine solution (cat. No.84957, Sigma) diluted 1:10 with sterile PBS (Ca^{2+}/Mg^{2+}-free). The poly-L-ornithine solution (1 ml) was kept on the cover slips for 10-15 minutes. Thereafter the cover slips were washed one time with PBS and the solution completely removed. Finally the cover slips was dried before seeding the cells.

3.4.2 DAPI staining of nuclear DNA

The DAPI staining of nuclei was used to distinguish alive cells from dead cells of which the nuclei are smaller, condensed and fractionated.

4´-6´-Diamidino-2-phenylindole (DAPI) forms fluorescent complexes with natural double-stranded DNA. DAPI binds to the minor groove of the DNA strand, the interaction being stabilized by hydrogen bonds between DAPI molecules and base pairs. After seeding, primary beta cells were cultured for 24 hours before treatment. After cells were treated for 1-2 days, DAPI stainging was performed in tissue culture. After removing the culture medium with test substances, cells were fixed and permeabilized 2 ml DAPI (0.1µg/ml) solution per dish was added and kept in dark for 10 minutes. The dishes were washed 1 time with PBS. Then the cells were washed with nomal water and dried with paper towel. At last moviol 20 µl was put in the middle of the object slides just before the cover slip was placed on the moviol drop (cells are between glass and slip). Then all slides were stored in a dark box until measurement of

fluorescence with a fluorescence microscope.

3.5 TUNEL (terminal dUTP nick-end labelling) assay of apoptotic cells

Terminal dUTP nick-end labelling (TUNEL) assay specifically detects DNA fragmentation which is characteristic for apoptotic cells. One of the hallmarks of late stage apoptosis is the fragmentation of nuclear chromatin which results in a multitude of 3´-hydroxyl termini of DNA ends. This property can be used to identify apoptotic cells by labelling the DNA breaks with fluorescent-tagged deoxyuridine triphosphate nucleotides (Fluorescein-dUTP). The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes a template-independent addition of deoxy-ribonucleoside triphosphates to the 3´-hydroxyl termini of generated DNA strands. Non-apoptotic cells do not incorporate much of the F-dUTP because of the absence of these exposed 3´-hydroxyl DNA ends and remain therefore unstained.

For TUNEL assay a In situ cell death detection kit, Fluorescein (cat.no. 11684795910, Roche Diagnostics) was used. The incorporated fluorescein-tagged nucleotides were detected by fluorescence microscopy, using an excitation of 488 nm and emission of 515-565 nm.

After 1 to 3 days of treatment, cells were prepared for TUNEL-staining, following the operating protocol provided by the kit. Shortly, the culture medium was removed and cells were fixed for 60-90 minutes in ice-cold paraformaldehyde (4% in PBS). After fixation, cells were washed twice with PBS and permeabilised for 2 minutes with 0.1% ice-cold Triton X-100 in 0.1% sodium citrate-containing PBS. During this step dishes must be kept on ice. The dishes then were washed twice with cold PBS and dried out. The TUNEL reaction mixture was carefully droped on the cells (50 µl each), the dishes were covered with parafilm and incubated 60 minutes at 37°C in the dark. After staining, TUNEL reaction mixture was removed and the dishes were washed twice with PBS. Thereafter all nuclei were stained with DAPI, and the samples examined under an inverted fluorescence microscope (Zeiss, Germany) by using a 10x objective, an excitation of 488 nm and an emission of 515-565 nm. The fluorescent green spots (TUNEL staining) in the microscopic field represent the apoptotic nuclei. DAPI staining was visualized with UV light using an excitation of 358 nm and emission of 461 nm.

3.6 Immofluorescent staining of cellular ceramide

The cells were cultured as mentioned before. After 24 hours the culture medium was replaced by medium without FCS as control group or by medium containing amyloid 0.1 µM. The dishes were kept in 37° C incubater for 30 min. Then the solution was sucked off and ice-cold paraformaldehyde (4% in PBS) added for 1 hour. After fixation the cells were washed twice with PBS and permeabilised for 2 minutes with 0.1% ice-cold Triton X-100 in 0.1% sodium citrate-containing PBS. During this step dishes were kept on ice. The dishes were then washed twice with cold PBS and dried out. To reduce non-specific binding PBS with 10% FCS was added to each dish and the dishes kept at RT for 45 minutes. Then cells were incubated with anti-ceramide antibody (monoclonal antibody against ceramide mouse IgM, ALEXIS, Germany). A drop of 100 µl (10 µl Ab added to 90 µl PBS containing 10% FCS) anti-ceramide antibody was placed on to the cells and kept in dark overnight at 4°C. The next day, the cells were washed 3 times with PBS containing 10% FCS (each wash-time 10 min). Then samples were stained with 2^{nd} antibody (Alexa Fluor 488 anti mouse IgG , Invitrogen, U.K). The cells were incubated with 100 µl 2^{nd} antibody (diluted in 1:400 with PBS containing 10% FCS) and kept for 1 h at RT. After washing, samples were stained with DAPI and the cover slips embedded with Moviol. Ceramide formation was analysed under a fluorescent microscopy (HAL100, Zeiss, Germany) using an excitation wavelength of 495-519 nm.

3. 7 Data analysis and statistics

Data are presented as means ± SEM. ANOVA for multiple groups and Student's t-tests were used for statistical analyses as appropriate. *P* values < 0.05 was accepted to indicate statistical significance.

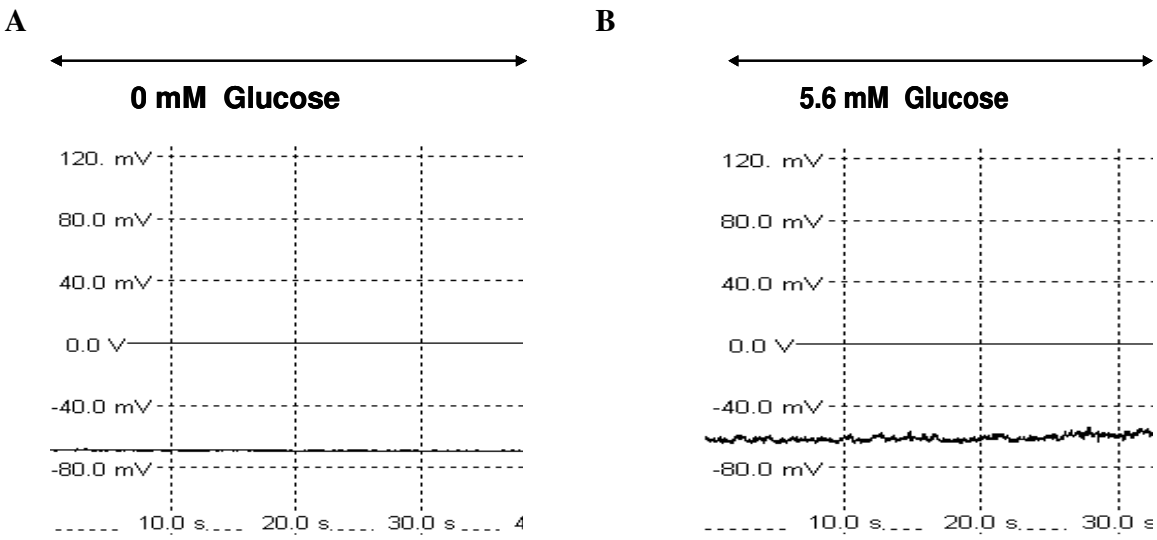
4 RESULTS

4.1 Electrical activity in primary mouse single islet cell

4.1.1 Glucose-induced electrical activity in pancreatic beta cells.

To evaluate how the primary islet cell population was responsive to glucose, glucose-induced electrical activity was measured after 2-4 days of tissue culture of the cells using the amphotericin B technique. This allows to quantify the number of cells responsive to glucose and to distinguish beta cells from non-beta cells.

Pancreatic islet cells are excitable cells that display electrical activites when stimulation occurs. It is well established that the metabolism of nutrient secretagogues such as glucose is essential for their action–i.e. the depolarization of the beta cell and the initiation of electrical activity (Henquin et al.1984). At glucose concentrations below 3 mM, which do not elicit insulin secretion, the beta cell is electrically silent with a resting membrane potential of about - 70 mV (see Fig.14A). Raising the external glucose produces a slow depolarization whose extent depends on concentration of the sugar (see Fig.14B). At glucose concentrations higher than 7 mM, that stimulate insulin release, depolarization becomes sufficient to reach the threshold potential (~ - 50 mV) at which electrical activity is initiated (see Fig. 14C). The individual action potentials are shown in more detail in Fig. 14D.



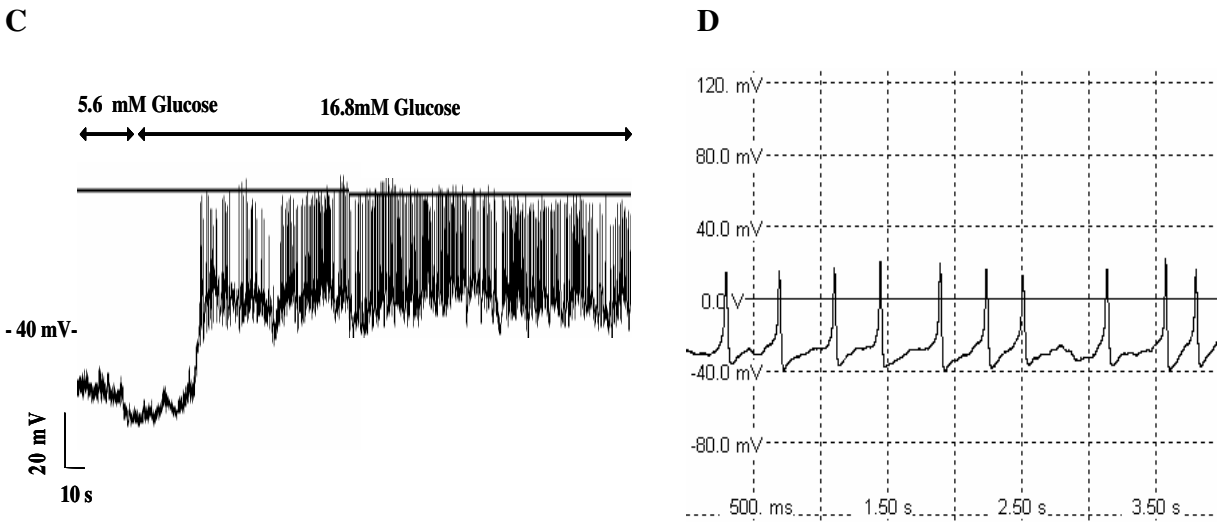


Fig. 14 : Glucose-induced electrical activity in pancreatic beta-cells.

A: At 0 mM glucose beta cells are electrically silent with a resting membrane potential of about - 70 mV (upper left panel). B: At 5.6 mM glucose beta cells slowly depolarize to about – 60 mV (upper right panel). C: Following the increase of glucose concentration to 16.8 mM was sufficient for the cell to depolarize of about -40 mV that reaching the threshold potential to initiate action potential. The plateau of action potential is sustained and continuous (lower left panel). D: Individual action potentials are shown with higher time resolution: the duration of each action potential is about 200 ms, the peak voltage of the action potential reached +20 mV. The plateau potential was ~ - 40 mV (recording of a cell at 11.2 mM glucose; lower right panel).

Table 6 summarizes the glucose-responsiveness of islet cells. These data show that 78% of the islet cells are responsive to glucose. The elevation of glucose from 5.6 mM to either 11.2 mM or 16.8 mM initiated action potentials from a similar plateau phase (- 38.50 ± 1.53 mV and -38.82 ± 1.07 mV; n= 26 and 38) (see Tab. 6). After rising the glucose concentration, action potentials were only initiated after 1 minute. This delay reflects the time that is needed for the cell to metabolise glucose and to rise cytosolic ATP concentration.

Tab. 6 : Islet cells glucose-responsive experiments.

	Rate	N	Mean (Voltage: mV)	SE	Initiating time (seconds)	Time to plateau (seconds)
Total experiments of glucose responses		213				
Total cells examined		82				
Measurements at 5.6mM glucose		82	-52.94	1.09		
Non-glucose responsive cells at 5.6mM glucose		18	-44.86	2.38		
Glucose responsive cells at 5.6mM glucose		64	-55.16	1.15		
Measurements at 11.2mM glucose		32	-41.32	1.51		
Measurements at 16.8mM glucose		50	-44.2	1.62		
Total non-glucose-responsive cells		18	-61.25	1.20		
Total glucose responsive cells		64				
Cells response to 11.2mM glucose		26	-38.50	1.53	66.92±7.37	97.31±10.09
Cells response to 16.8mM glucose		38	-38.82	1.07	62.63±6.93	103.82±10.01
Islet cells initiate action potential to glucose (>8 mM)	78.05%	82				

4.1.2 Tolbutamide induces action potential in primary mouse single islet cell.

Certain pharmacological agents such as sulphonylureas block the K_{ATP} channel directly. Tolbutamide depolarized beta-cell and initiate electrical activity within few seconds (Sturoess et al.1985) (see Fig. 15).

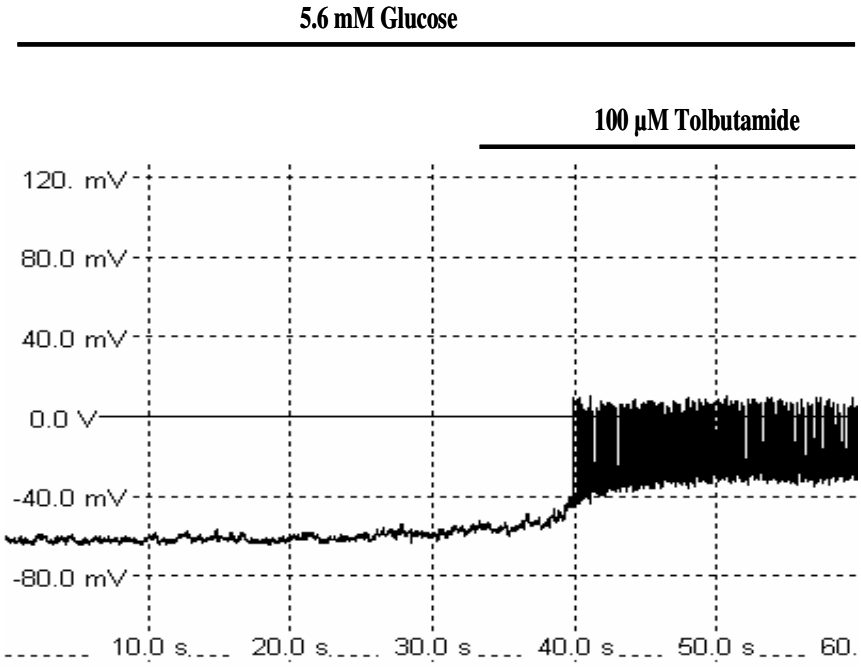


Fig.15: Tolbutamide induces action potentials in primary mouse single islet cell. During AmB perforated whole-cell recording, addition of 100µM Tolbutamide to 5.6 mM glucose rapidly elicits depolarization and continuous action potential.

In general, tolbutamide-induced action potentials were stable and sustained. The plateau phase was at about – 32.82 ± 0.73 mV (n= 93). The initiation of action potential was rapid and started 3.63 ± 0.24 seconds (n= 93) after addition of tolbutamide.

4.2 Regulation of membrane potential and current by hormones and peptides

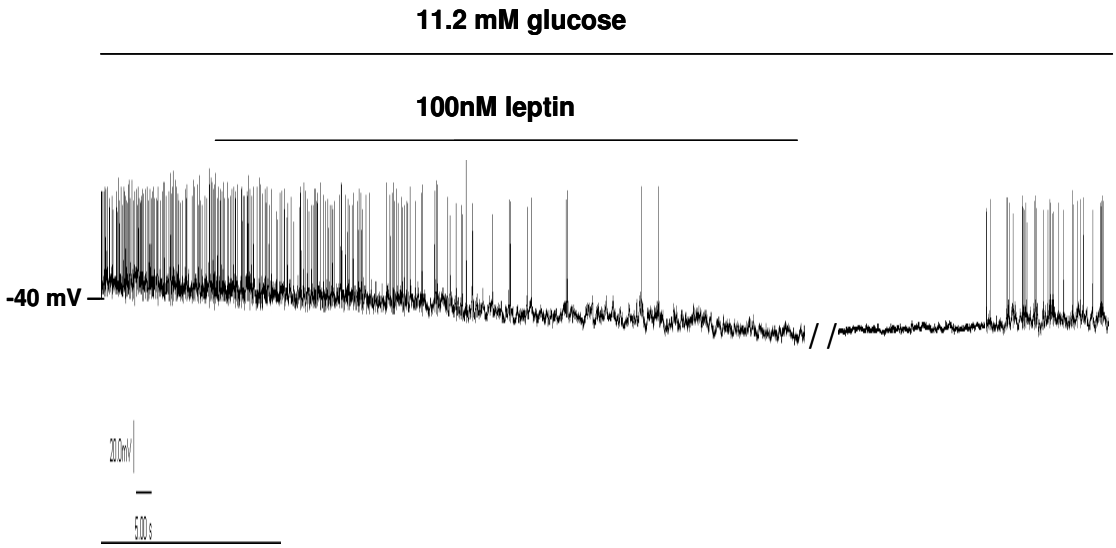
4.2.1 Leptin regulates membrane potential and current via a PI 3-kinase-dependent process in mouse islet cells.

It has been shown that injection of leptin into wild type and ob/ob mice significantly decreases body weight and serum concentrations of glucose and insulin (Halaas et al.1995; Pelleymounter et al.1995). To examine the direct effect of leptin on channel activity induced by glucose and to study the involvement of phosphoinositide 3-kinase (PI3K), leptin was exogenously added to the perfused bath solution in the absence and presence of PI3K inhibitors LY294002 and wortmannin.

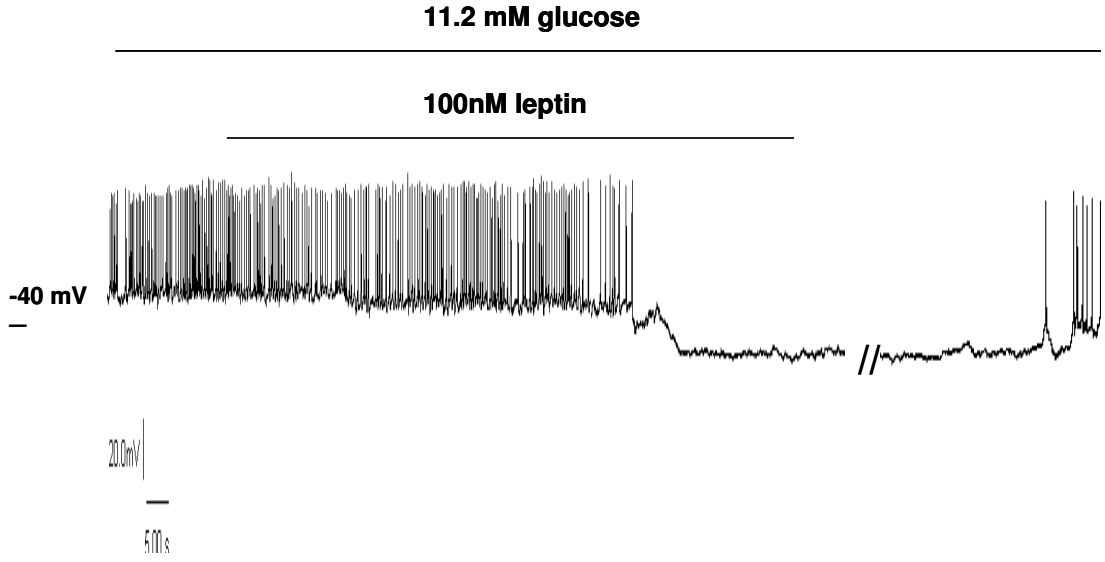
Under current-clamp conditions with AmB in the pipette solution (formation of perforated whole-cell patch clamp), the mean membrane potential of mouse primary pancreatic beta cell was -37.11 ± 0.37 mV (n=38) at 11.2 mM glucose. The application of leptin (100 nM) resulted in a hyperpolarization of the plasma membrane to -54.12 ± 2.31 mV (n=9; $P<0.001$; Fig. 16A1, A2 and B). After 5 minutes wash out leptin from the bath solution, membrane potential partially reversed (n=4; -42.58 ± 1.27 mV). The hyperpolarization induced by leptin was slow, started 2-3 minutes after addition of leptin and achieved the maximum after 3-5 minutes. The examination of the voltage-clamped currents (Fig. 16C1 and C2) revealed that prior to the application of leptin (100nM), the control peak outward current density was 72.11 ± 9.14 pA/pF and increased after addition of leptin to 88.84 ± 17.29 pA/pF (n=4; $P=0.1518$; Fig. 16D1). At the same time the conductance of the cells increased with leptin from 0.65 ± 0.04 nS to 0.83 ± 0.16 nS (n=4; $P=0.1574$; Fig. 16D2). The application of inhibitors of PI 3K, LY294002 (10 µM; n=6; Fig. 16E and 16F) had no significant effect on the resting membrane potential but prevented the leptin-induced hyperpolarization. The perfusion with LY and leptin was continued for at least 5 minutes.

These data suggest that leptin exerts its hyperpolarizing effect on membrane potential through the activation of PI3K pathway.

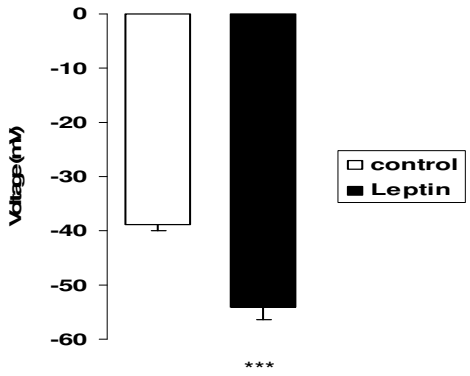
A 1


11.2 mM glucose
100nM leptin
-40 mV
20.0mV
5.00 s

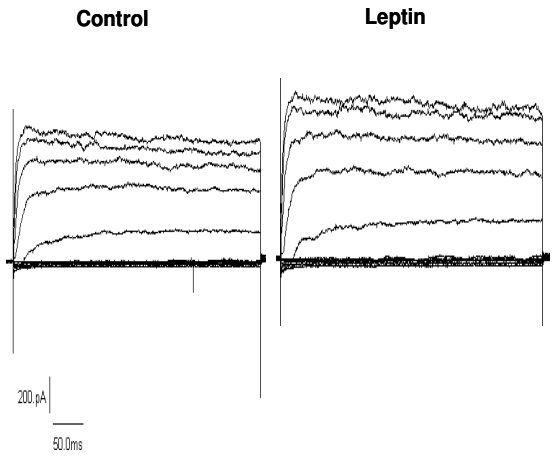
A 2


11.2 mM glucose
100nM leptin
-40 mV
20.0mV
5.00 s

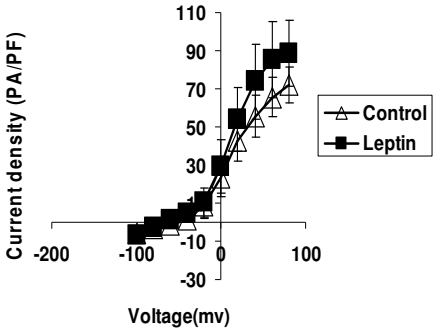
B


0
-10
-20
-30
-40
-50
-60
control
Leptin
***

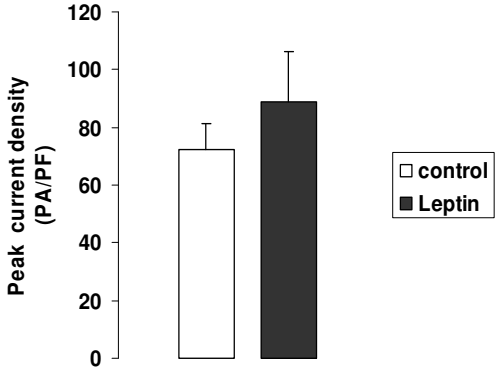
C 1


Control
Leptin
200 pA
50.0ms

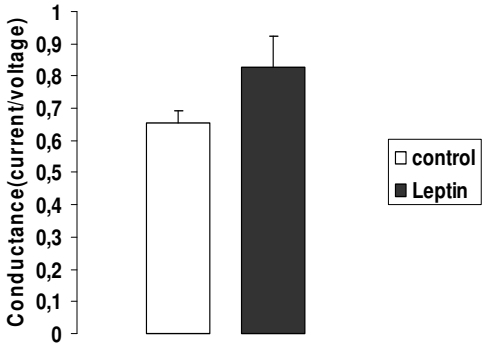
C 2


110
90
70
50
30
-10
-30
-200
-100
0
100
Current density (PA/PF)
Voltage(mv)
Control
Leptin

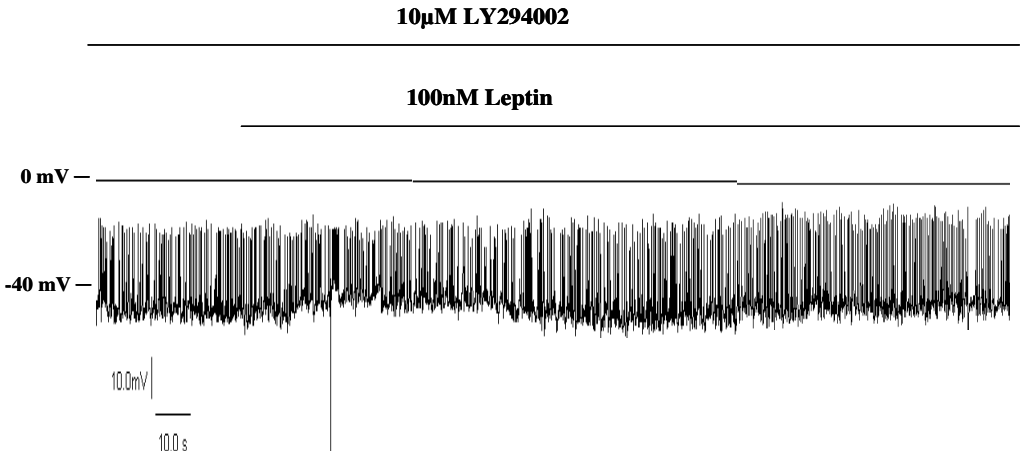
D 1


120
100
80
60
40
20
0
Peak current density (PA/PF)
control
Leptin

D 2


1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
Conductance(current/voltage)
control
Leptin

E



F

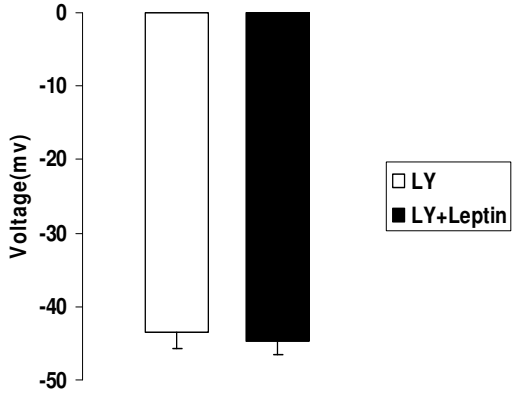


Fig.16: Leptin regulates membrane potential and current via a PI 3-kinase-dependent process in mouse islet cells. A1: Representative AmB perforated whole-cell current clamp recording of a primary mouse islet cell. The application of leptin (100 nM) resulted in a slow change of membrane potential from -37 mV to -50 mV. Membrane potential decreased after 2.5 minutes and achieved a maximum. After 4 minutes wash-out of the leptin from the bath, the effect was reversed. The break corresponds to 3 min. A2: Using the same protocol as above, application of leptin resulted in a hyperpolarization from -39 mV to -60 mV. It lasted 2 minutes before initiation of response and it achieved a maximum after 3 minutes. After 4 minutes wash-out of the leptin from the bath, the effect was reversed. The break corresponds to 3 min. B:

Arithmetic means ± SEM (n=9) of membrane potential (mV) prior to (white bar) and after exposure of the cells to leptin (100 nM; black bar). *** indicates great significant difference between leptin and control (P<0.001; two-tailed paired t-test). C1: Original tracings of perforated whole-cell recording of membrane current prior to (C1 left measurement) and after addition of leptin (100 nM; C1 right measurement). C2: I-V relations of mean current prior to (open triangles) and after exposure of mouse islet cells to leptin (100 nM; closed squares). D1: Arithmetic means ± SEM (n=4) of peak current density (pA/pF) prior to (white bar) and after exposure of the cells to leptin (100 nM; black bar). D2: Arithmetic means ± SEM (n=4) of slop conductances (nS) of mouse islet cells prior to (white bar) and after exposure of the cells to leptin (100 nM; black bar). E: Original AmB perforated whole-cell current clamp recording of a primary mouse islet cell. Application of LY 294002 (10 μM) had no effect on the resting membrane potential. Application of leptin (100 nM) after exposure to LY 294002 failed to hyperpolarize mouse islet cells. F: Arithmetic means ± SEM (n=6) of membrane potential (mV) prior to (white bar) and after exposure of the cells to leptin (100 nM; black bar). LY 294002 prevented the leptin-induced hyperpolarization in mouse islet cells.

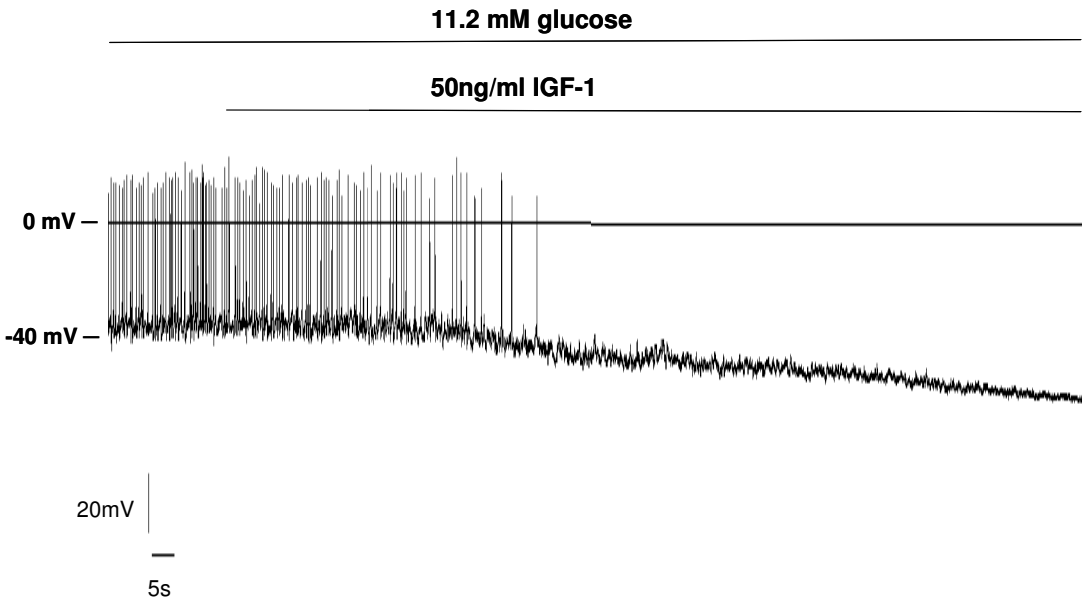
4.2.2 IGF-1 regulates membrane potential and current via a PI 3-kinase-dependent process in mouse islet cells

It has been shown that IGF-1 inhibits insulin secretion that has been increased by glucose and glucagonlike peptide 1 in an insulinoma cell line (Zhao et al.1997), in rat pancreas (Fürnsinn et al.1994; Leahy et al.1990) and also in isolated beta cells (Van et al.1990). As IGF-1 stimulates PI3K pathways, its effect on membrane potential was examined using the perforated whole cell patch clamp configuration as for leptin.

The mean membrane potential of mouse primary pancreatic beta cell in the presence of 11.2 mM glucose was -39.54 ± 2.08 mV(n=8). Following application of IGF-1 (50 ng/ml) the membrane slowly hyperpolarized to -53.32 ± 1.07 mV (n=8;P<0.001; Fig. 17A and B) in mouse single islet cell. The response to IGF-1 was initiated 3-5 minutes after application. This hyperpolarization was not reversed after 15 minutes after remove of IGF-1. The application of the PI 3-kinase inhibitors LY294002 (10 μM; Fig. 17C and D) had no significant effect on membrane potential but prevented IGF-1-induced hyperpolarization (LY -39.60 ± 1.42mV vs LY with IGF-

1 -41.06 ± 2.24mV; n=5 respectively). The perfusion of LY and IGF-1 in combination was continued at least 5 minutes.

A



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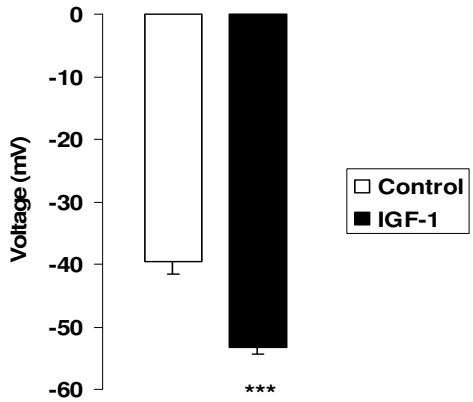

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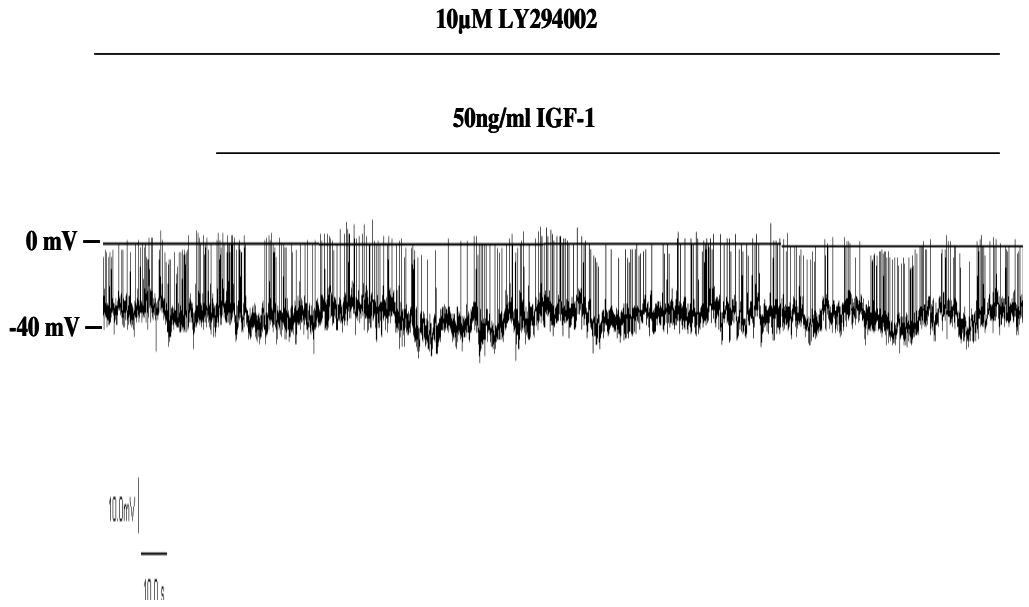

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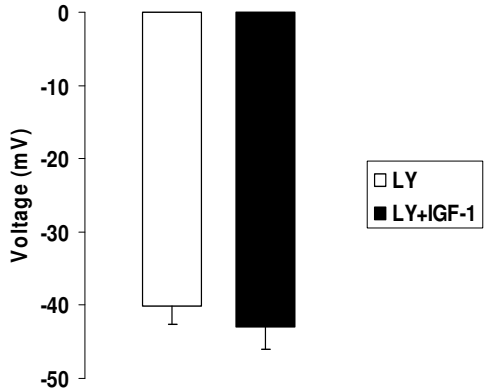


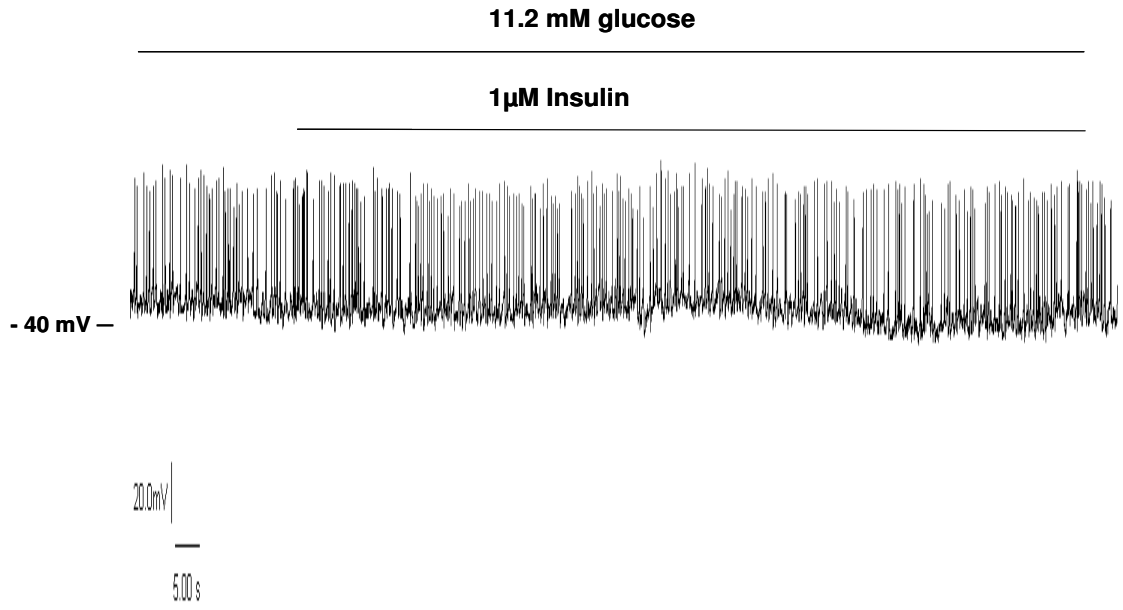
Fig.17: IGF-1 reduced membrane activity via a PI 3-kinase-dependent process in mouse islet cells. A: Representative AmB perforated whole-cell current clamp recording of a primary mouse islet cell after application of IGF-1 (50 ng/ml). The membrane potential hyperpolarized from -36 mV to -57 mV. The response to IGF-1 started 2.5 minutes after application. After 15 minutes wash-out of IGF-1 from the bath, the effect was not reversed. B: Arithmetic means ± SEM (n=8) of membrane potential (mV) prior to (white bar) and after exposure of the cells to IGF-1 (50 ng/ml; black bar). *** indicates very significant difference between control and IGF-1 (P<0.001; two-tailed paired t-test). C: Original recording of a mouse islet cell. Application of LY

294002 (10 µM) had no effect on membrane potential. Application of IGF-1 (50 ng/ml) after exposure to LY 294002 failed to hyperpolarize mouse islet cells. D: Arithmetic means ± SEM (n=5) of membrane potential (mV) prior to (white bar) and after exposure of the cells to IGF-1 (50 ng/ml; black bar). LY 294002 prevented the IGF-1-dependent hyperpolarization in mouse islet cells.

4.2.3 Exogenous insulin has no significant effect on whole-cell membrane potential in mouse islet cells

To examine whether insulin also hyperpolarizes beta cells, comparable experiments using the perforated whole-cell patch clamp have been performed. The mean membrane potential of mouse primary pancreatic beta cell was -33.50 ± 3.25 mV (n=3). Following application of exogenous insulin (1 µM) had no significant effect on membrane potential (-34.08 ± 3.52 mV; n=3; *P*>0.05; Fig. 18A and B) in mouse single islet cell. The perfusion of the exogenous insulin was continued at least 5 minutes.

A



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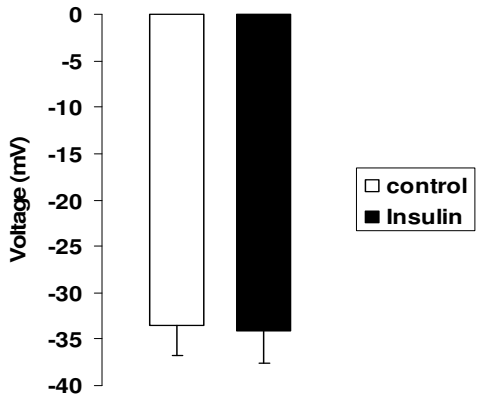


Fig. 18: Exogenous insulin has no significant effect on whole-cell membrane potential in mouse islet cells. A: Representative AmB perforated whole-cell current clamp recording of a primary mouse islet cell after application of exogenous insulin (1 µM) had no effect on membrane potential. B: Arithmetic means ± SEM (n=3) of membrane potential (mV) prior to (white bar) and after exposure of the cells to exogenous insulin (1 µM; black bar; $P>0.05$; two-tailed paired t-test).

4.2.4 Adrenaline regulates membrane potential and current via a PI 3-kinase-dependent and PDK1 process in mouse islet cells.

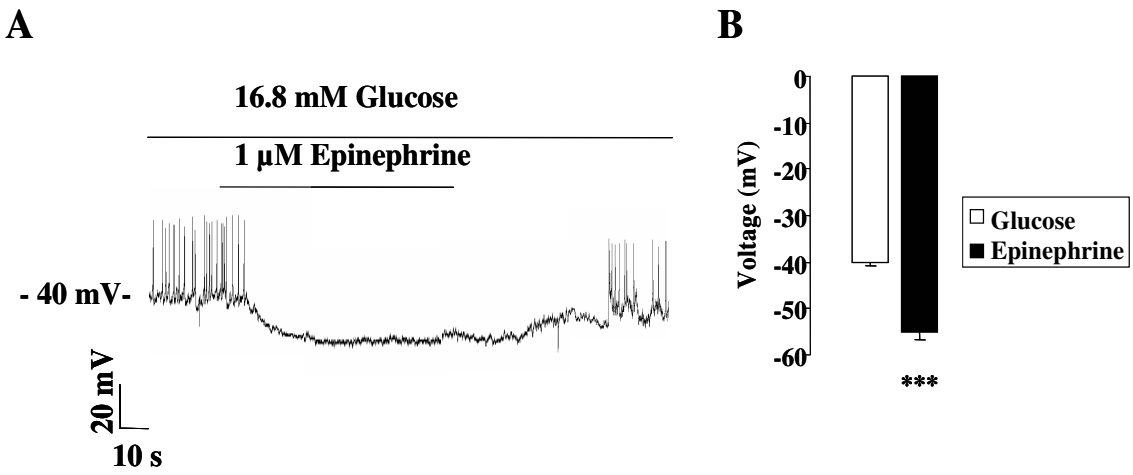
That adrenaline (or epinephrine) inhibits insulin secretion and hyperpolarizes beta cells, has been demonstrated decades ago (Fowden 1980; Ullrich et al.1984; Ullrich et al.1985; Ullrich et al.1988; Lacey et al.1993; Abel et al. 1996; Ullrich et al.1996; Lehr et al.1997; Kampermann et al.2000; Peterhoff et al.2003; Sieg et al.2004;). However, the mechanism of adrenaline induced hyperpolarization is still not completely understood. The following experiments were performed to examine whether adrenaline-induced hyperpolarization depends on PI3-kinase activity.

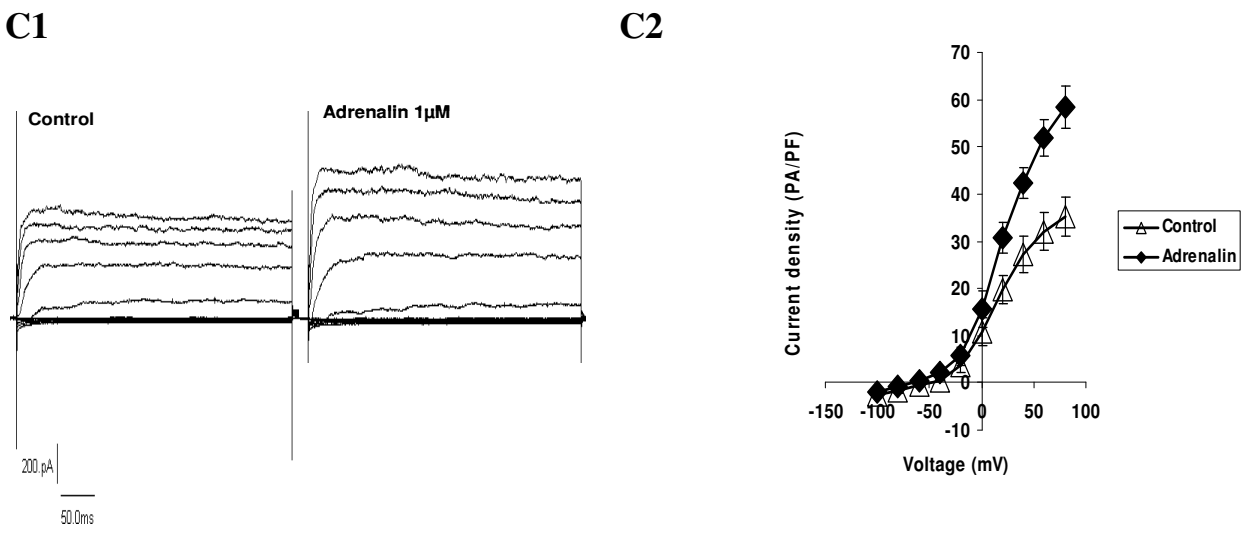
Under current-clamp conditions with AmB in pipette solution (formation of perforated whole-cell patch clamp) and in the presence of 16.8 mM glucose in bath solution, the mean membrane potential of mouse primary pancreatic beta cell was -38.82 ± 1.07 mV (n=38) and

action potential were generated. Following application of adrenaline (1 µM) resulted in a hyperpolarization to -55.24 ± 1.37 mV (n=16; $P<0.001$; Fig. 19A and B). 1-5 minutes after wash-out of adrenaline from the perfusion solution, in 12 out of 16 recordings the effect was reversed (n=12; -38.59 ± 2.00 mV; compares with adrenaline $P<0.01$; recording did not show here). The hyperpolarizing response of adrenaline was initiated 10-15 seconds after addition of adrenaline and a maximum was achieved after 20-30 seconds. Examination of the voltage-clamped currents (Fig. 19C1 and C2) show that prior to adrenaline application (1 µM), the control peak outward current density was 41.64 ± 4.61 pA/pF and following exposure to adrenaline a current was significantly increased to 58.46 ± 4.49 pA/pF (n=5; $P<0.01$; Fig. 19D). Application of the PI 3-kinase inhibitor LY294002 (10 µM; n=7; Fig. 19E and F) had no significant effect on membrane potential but prevented the adrenaline-induced hyperpolarization (LY -40 ± 2.1mV vs LY with adrenaline -42.1 ± 2.83mV; n=7 respectively; $P>0.05$). The perfusion of the combination of LY and adrenaline was continued at least 5 minutes. Application of another inhibitor of PI 3-kinase, wortmannin (100nM; n=7; Fig. 19G and H), also showed no significant effect on the membrane potential, but prevented the adrenaline-induced hyperpolarization (wortmannin -45.71 ± 3.62mV vs wortmannin with adrenaline -48.85 ± 3.7mV; n=7 respectively; $P>0.05$).

The hyperpolarizing effect of epinephrine was also observed in $pdk1^{wt}$ mouse islet cells (Fig. 19 I and J) but abrogated in $pdk1^{hm}$ mouse islet cells (Fig. 19 K and L). In the presence of 16.8 mM glucose application of epinephrine (1 µM) resulted in a significant ($P < 0.05$) hyperpolarization from -40.3 ± 3.6 mV to -53.4 ± 3.2 mV (n = 5) in $pdk1^{wt}$ mouse single islet cells (Fig. 19 I). In contrast, as shown in Fig. 19 I, the application of epinephrine (1 µM) in the presence of 16.8 mM glucose did not significantly ($P = 0.11$) hyperpolarize $pdk1^{hm}$ mouse islet cells (-41.5 ± 1.4 mV prior to and -44.6 ± 2.3 mV following epinephrine, n = 7) (Fig. 19 K and L).

These results suggest that adrenaline-induced hyperpolarization depends on PI3K and PDK1 activities.


A
16.8 mM Glucose
1 µM Epinephrine
- 40 mV-
20 mV
10 s
B
Voltage (mV)
0
-10
-20
-30
-40
-50
-60
***
Glucose
Epinephrine


C1
Control
Adrenalin 1µM
200 pA
50.0ms
C2
Current density (PA/PF)
70
60
50
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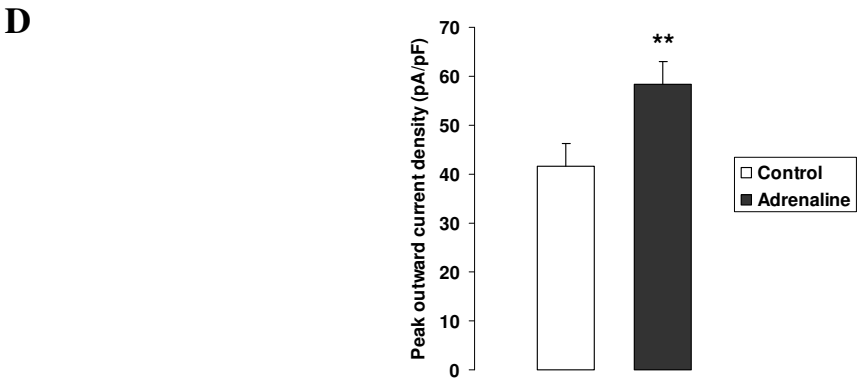
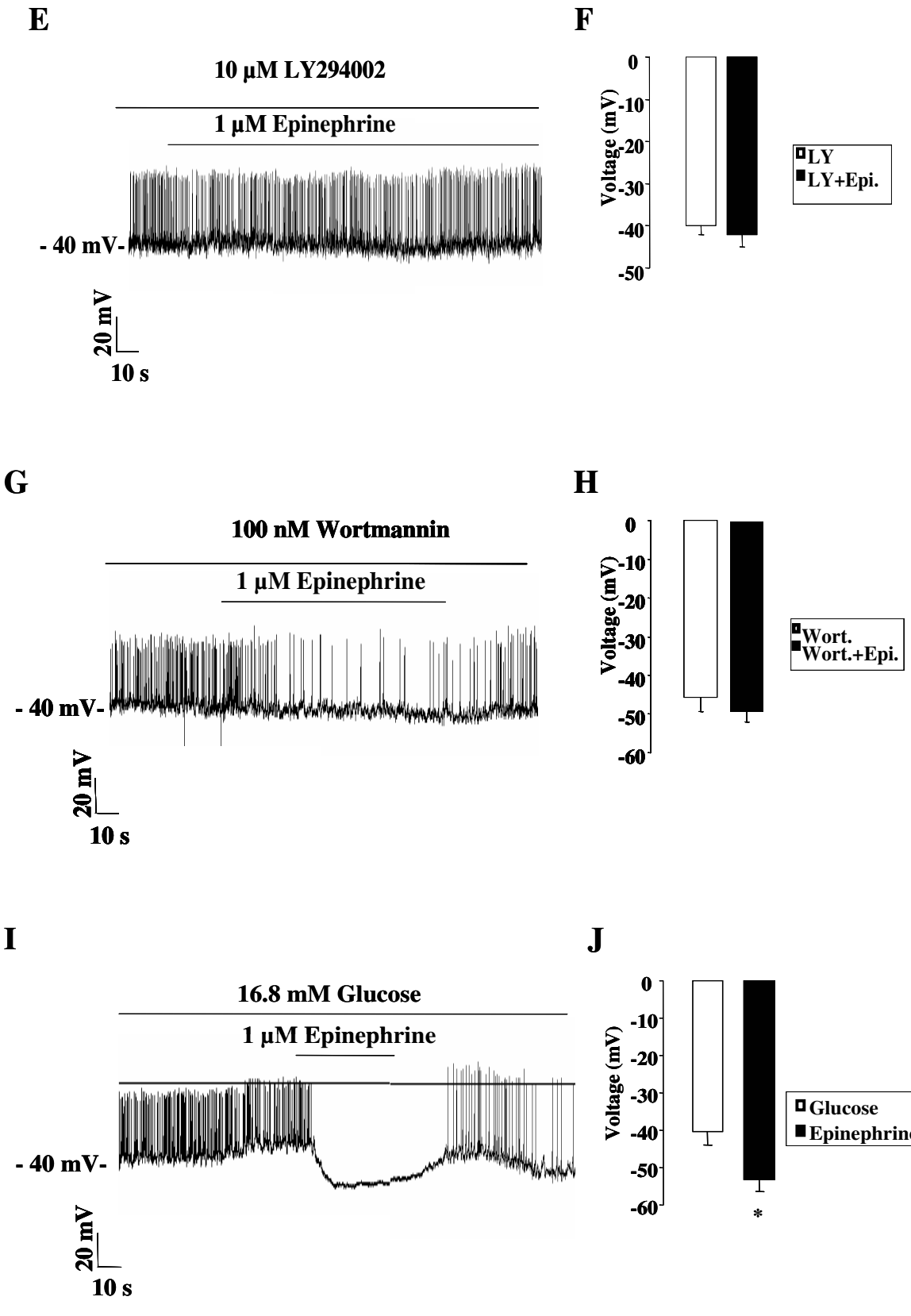
-10
-150
-100
-50
0
50
100
Voltage (mV)
Control
Adrenalin


D
Peak outward current density (pA/pF)
70
60
50
40
30
20
10
0
**
Control
Adrenaline


E
10 µM LY294002
1 µM Epinephrine
- 40 mV-
20 mV
10 s
F
Voltage (mV)
0
-10
-20
-30
-40
-50
LY
LY+Epi.
G
100 nM Wortmannin
1 µM Epinephrine
- 40 mV-
20 mV
10 s
H
Voltage (mV)
0
-10
-20
-30
-40
-50
-60
Wort.
Wort.+Epi.
I
16.8 mM Glucose
1 µM Epinephrine
- 40 mV-
20 mV
10 s
J
Voltage (mV)
0
-10
-20
-30
-40
-50
-60
*
Glucose
Epinephrine

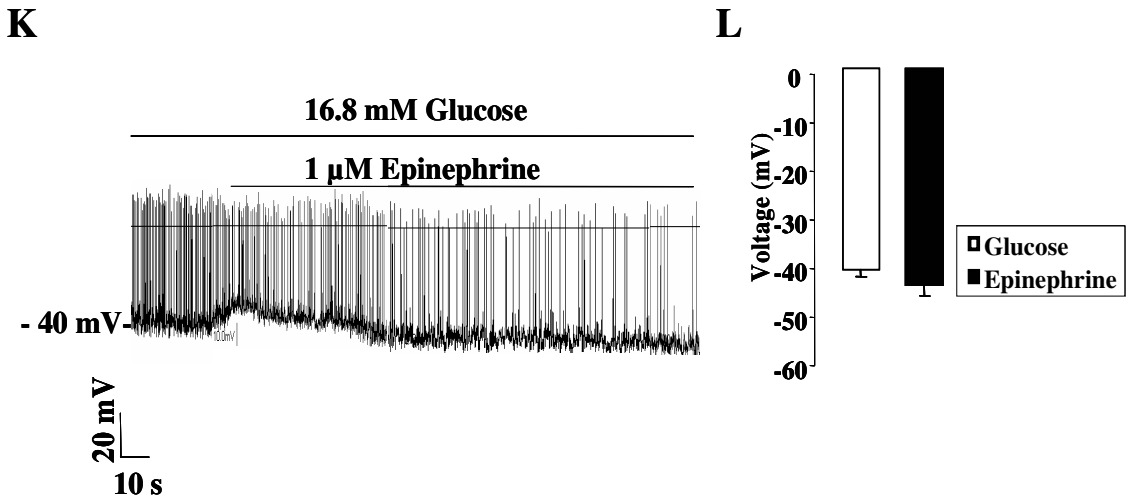


Fig. 19: Adrenaline regulates membrane potential and current via a PI 3-kinase-dependent and PDK1 process in mouse islet cells. A: Representative AmB perforated whole-cell current clamp recording of a primary mouse islet cell after application of adrenaline (1 µM) induced hyperpolarization from -41 mV to -59 mV. The response started 10 seconds after addition of adrenaline and was maximal at the 18 seconds. After 1 minute wash-out of the adrenaline from the bath, the inhibition of action potential was reversed. B: Arithmetic means ± SEM (n=16) of membrane potential (mV) prior to (white bar) and after exposure of the cells to adrenaline (1 µM; black bar). *** indicates very significant difference between control and addition of adrenaline (P<0.001; two-tailed paired t-test). C1: Original tracings of perforated whole-cell recording of membrane current prior to (C1 left measurement) and after addition of adrenaline (1 µM; C1 right measurement). C2: I-V relations of mean current prior to (open triangles) and after exposure of mouse islet cells to adrenaline (1 µM; closed squares). D: Arithmetic means ± SEM (n=5) of peak current density (pA/pF) prior to (white bar) and after exposure of the cells to adrenaline (1 µM; black bar). ** indicates significant difference between control and addition of adrenaline (P<0.01; two-tailed paired t-test). E: Original recording of a islet cell. Application of LY 294002 (10 µM) had no effect on membrane potential. Application of adrenaline (1 µM) in the presence of LY 294002 failed to hyperpolarize mouse islet cells. F: Arithmetic means ± SEM (n=7) of membrane potential (mV) prior to (white bar) and after exposure of the cells to adrenaline (1 µM;

black bar). There is no significant difference between application of LY and adrenaline combined with LY. LY 294002 prevented the adrenaline-induced hyperpolarization in mouse islet cells. G: Original recording of a islet cell. Application of Wortmannin (100 nM) had no effect on membrane potential (n=7). Application of adrenaline (1 µM) in the presence of Wortmannin (100 nM) failed to hyperpolarize mouse islet cells. H: Arithmetic means ± SEM (n=7) of membrane potential (mV) prior to (white bar) and in the presence of the cells to adrenaline (1 µM; black bar). There is no significant difference between application of Wortmannin (100 nM) and adrenaline in the presence of Wortmannin (*P*=0.08). Wortmannin (100 nM) prevented the adrenaline-induced hyperpolarization of mouse islet cells. I: Original tracings of cell membrane potential following an increase of extracellular glucose concentration to 16.8 mM and subsequent addition of 1 µM epinephrine in islet cells from PDK1 wild type littermates (*pdk1*^{wt}). J: Mean cell membrane potential (± SEM, n=5) at 16.8 mM glucose concentration in the absence (white bars) and presence (black bars) of 1 µM epinephrine in islet cells from PDK1 wild type littermates (*pdk1*^{wt}). * indicates significant difference from respective value in the absence and presence of epinephrine (paired T-Test. $P \leq 0.05$). K: Original tracings of cell membrane potential following an increase of extracellular glucose concentration to 16.8 mM and subsequent addition of 1 µM epinephrine in islet cells from PDK1 hypomorphic mice (*pdk1*^{hm}). L: Mean cell membrane potential (± SEM, n=7) at 16.8 mM glucose concentration in the absence (white bars) and presence (black bars) of 1 µM epinephrine in islet cells from PDK1 hypomorphic mice (*pdk1*^{hm}).

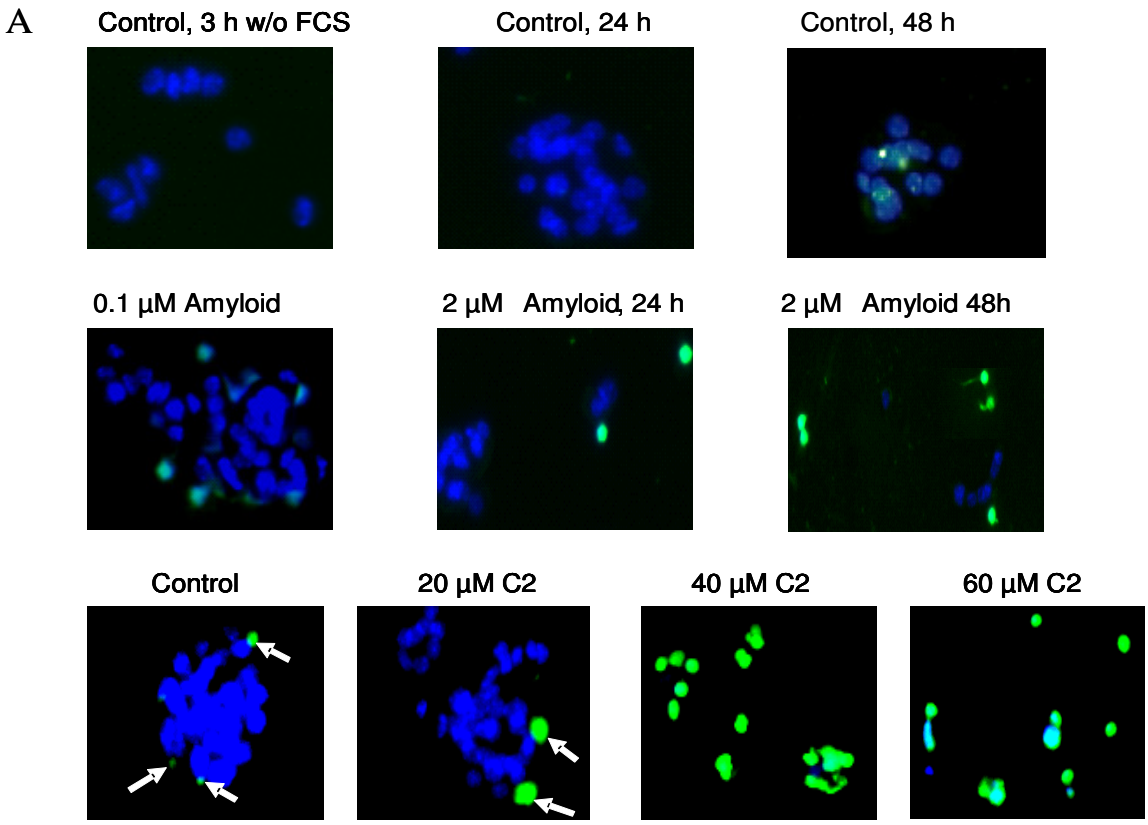
4.3 Effects of amyloid and ceramide on apoptosis and activity of K_V channel in mouse pancreatic beta cells

Amyloid peptides have been known to interfere with cell survival. As in some cells apoptotic death is paralleled by ceramide dependent alterations of plasma membrane ion channel activity. The present study elucidated the effects of amyloid on ceramide formation and ion channel activity in mouse islets.

4.3.1 Effect of amyloid beta fragment $A\beta_{1-42}$ and C_2-ceramide on apoptosis of islet cells

Isolated islet cells treated with amyloid beta fragment $A\beta_{1-42}$ for 3 hours underwent apoptotic cell death (Fig.20A and B). The effect of amyloid was significant at a concentration of 0.1 µM in the absence of serum whereas under standard culture conditions in the presence of serum higher concentrations of amyloid (2 µM) were required for a statistically significant effect (Fig. 20C). When C_2-ceramide at concentrations higher than 40 µM was added into standard culture medium 90 % of the cells died within 2 d. However, lower concentrations of C_2-ceramide (20 µM) were without any significant effect (Fig .20A, D).

These observations show that ceramide and amyloid induce cell death of isulin-secreting cells.



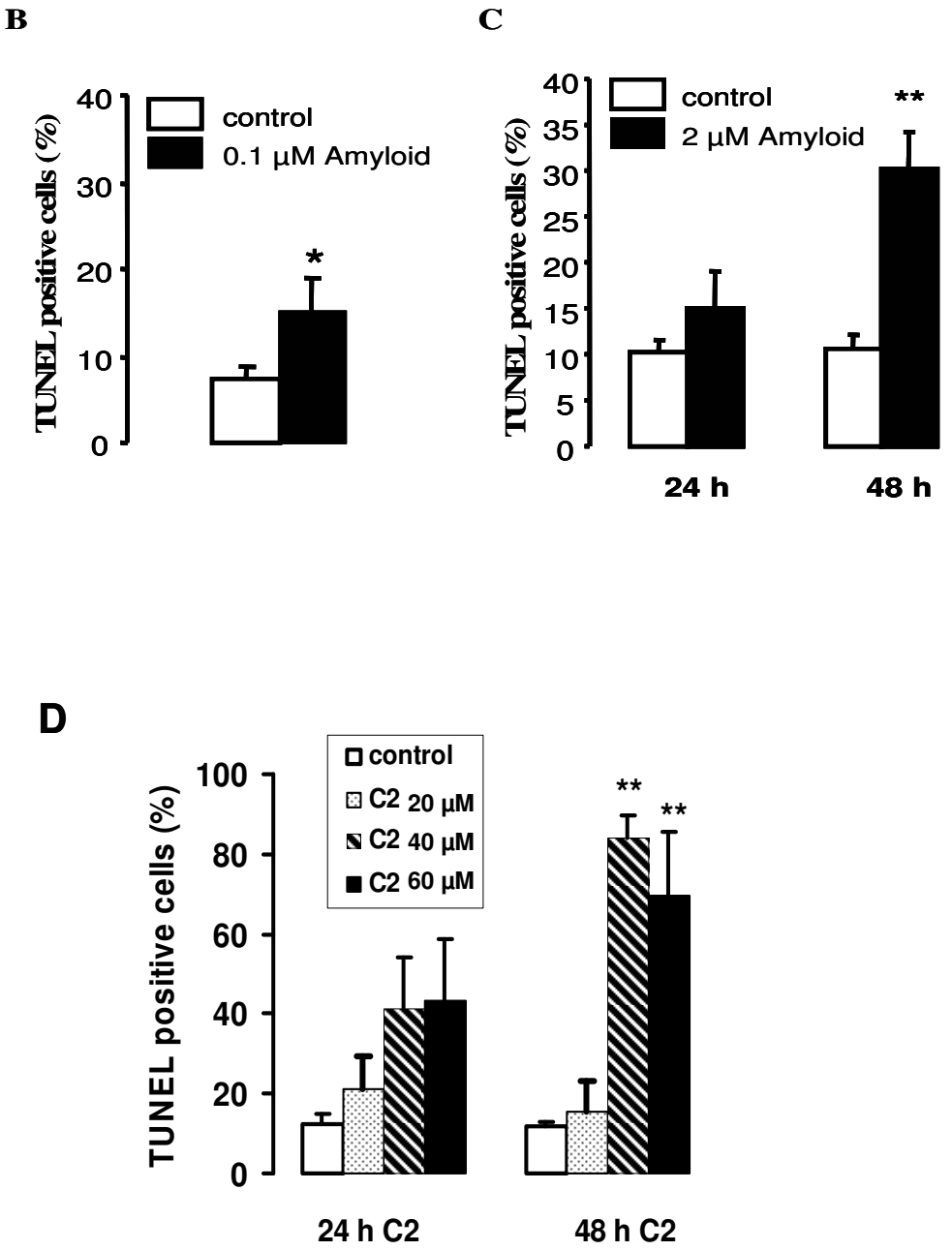


Fig. 20: Effect of amyloid beta fragment Aß$_{1-42}$ and C$_2$-ceramide on apoptosis of pancreatic beta cells. A. Representative images illustrating fluorescent DAPI+TUNEL staining of pancreatic beta cells incubated for 0.1 µM for 3 h, 2 µM for 24h , 2 µM for 48 h with (middle 3 panels) or without (upper 3 panels) amyloid peptide Aß$_{1-42}$, or for 48 h with (lower right 3

panels) or without (lower left panel) C_2-ceramide (20-60 µM). White arrows and green stained cells indicate TUNEL positive cells which suggest apoptotic cells. All nuclei were stained blue by DAPI. B. Arithmetic means ± SEM (n = 4) of fractionated nuclei in pancreatic beta cells incubated for 3 h with (black bar) or without (open bar) 0.1 µM amyloid peptide $Aß_{1-42}$. * indicates significant difference ($P< 0.05$; two-tailed unpaired t-test). C. Arithmetic means ± SEM (n = 4) of fractionated nuclei in pancreatic beta cells exposed 24 h (left bars) or 48 h (right bars) with (black bar) or without (open bar) 2 µM amyloid peptide $Aß_{1-42}$ (ANOVA, $P< 0.01$) . D. Arithmetic means ± SEM (n = 4) of fractionated nuclei in pancreatic beta cells incubated for 24 h (left bars) or 48 h (right bars) in the absence (control, white bars) or in the presence of different concentrations of C_2-ceramide (20 µM dotted bar, 40 µM hatched bar and 60 µM black bar). ** indicates significant difference from control (ANOVA, $P< 0.01$).

4.3.2 Effect of amyloid beta fragment $Aß_{1-42}$ on ceramide formation in pancreatic beta cells

To further analyse whether amyloid peptide $Aß_{1-42}$ stimulates ceramide production in isolated islet cells, the formation of ceramide was visualized by immunofluorescent labelling with specific ceramide antibodies. In control cells ceramide was visualized mainly at the periphery of the cells (Fig. 21). Treatment of cells with 0.1 µM amyloid for 30 min yielded a marked increase of cellular ceramide staining again with stronger staining at the cell periphery. These observation suggest that amyloid peptide $Aß_{1-42}$ may induce apoptotic cell death through the formation of ceramide.

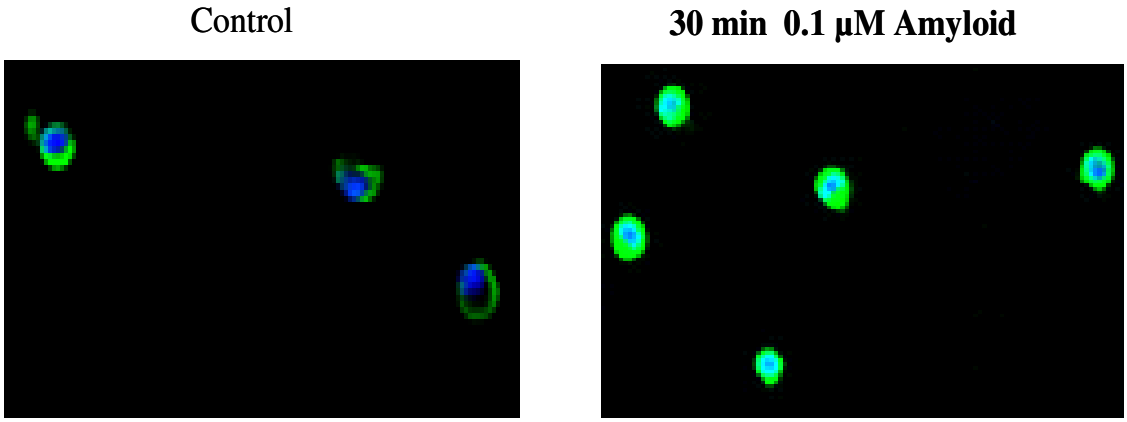


Fig. 21: Effect of amyloid beta fragment Aß$_{1-42}$ on ceramide formation in pancreatic beta cells. Fluorescence microphotograph of ceramide abundance in pancreatic beta cells prior to (left panel) and 30 min following (right panel) treatment with amyloid peptide Aß$_{1-42}$ (0.1 µM).

4.3.3 Effect of amyloid beta fragment Aß$_{1-42}$ on apoptosis in ASM-KO mouse pancreatic islet cells.

Ceramide formation underneath the plasma membrane may be a result of the activation of acid sphingomyelinase (ASM) that cleaves membrane sphingomyeline. To examine whether activation of ASM is involved in amyloid-induced apoptotic cell death, the effect of amyloid was examined in an islet cell preparation isolated from ASM knockout mice. Even high concentrations of amyloid were unable to increase apoptotic cell death over the basal rate of 7 % in ASM-KO islet cells (Fig. 22). These observations strongly suggest that amyloid induces apoptotic cell death via activation of ASM amd subsequent increase of ceramide production.

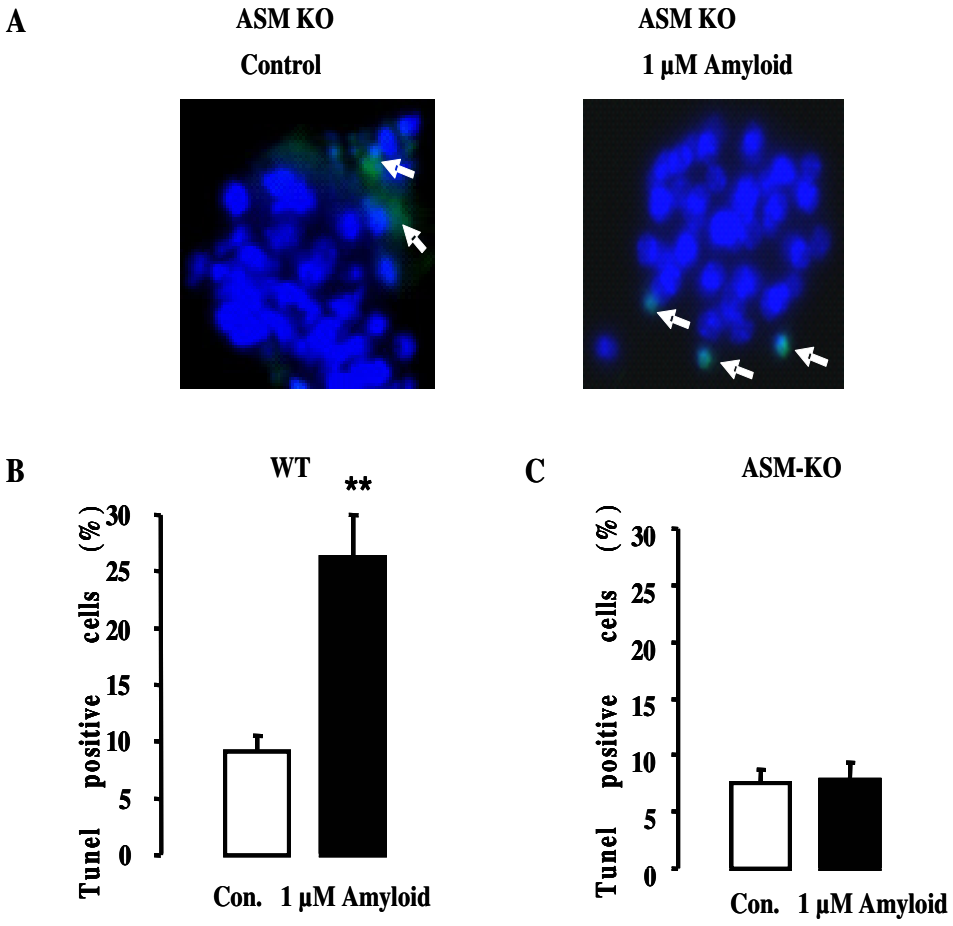
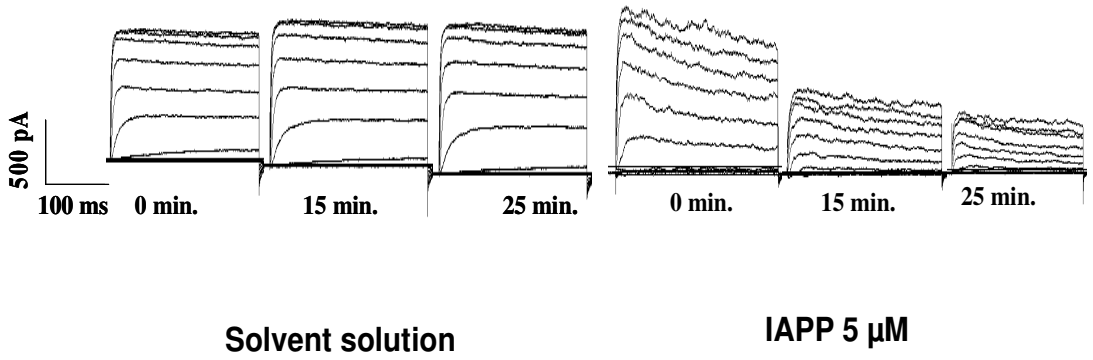


Fig. 22: Effect of amyloid beta fragment Aß1-42 on apoptosis in ASM-KO mouse pancreatic islet cells. A: Representative images illustrating fluorescent DAPI+TUNEL staining of ASM-KO mouse pancreatic islet cells without treatment (left panel) and 24 h after (right panel) treatment with 1 µM amyloid (white arrows denote TUNEL positive cells). B: Arithmetic means ± SEM (n = 4) of fractionated nuclei in wild type mice pancreatic beta cells incubated for 24 hours with (black bar) or without (open bar) 1 µM amyloid peptide $Aß_{1-42}$.** indicates significant difference ($P< 0.01$; two-tailed unpaired t-test). C: Arithmetic means ± SEM (n = 4) of fractionated nuclei in ASM-KO mice pancreatic beta cells incubated for 24 hours with (black bar) or without (open bar) 1 µM amyloid peptide $Aß_{1-42}$.

4.3.4 Effect of islet amyloid polypeptide (IAPP, 5 µM) on K_v channel current in WT islet cells

As apoptotic cell death is accompanied by changes in ion channel activity, the effect of amyloid on whole cell ion current was examined. The administration of islet amyloid polypeptide (IAPP, 5 µM) led to a significant decrease of the whole cell outward current within 15 min from 128.80 ± 9.84 to 87.28 ± 10.30 (pA/pF, n = 6, $P< 0.001$) and within 25 min the outward current was reduced to 78.08 ± 10.03 (n = 6, $P< 0.001$) (Fig. 23).

A



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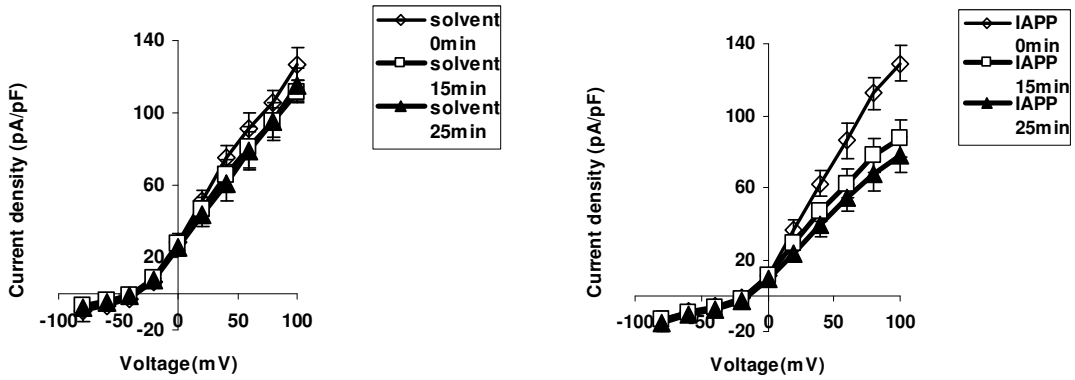


c

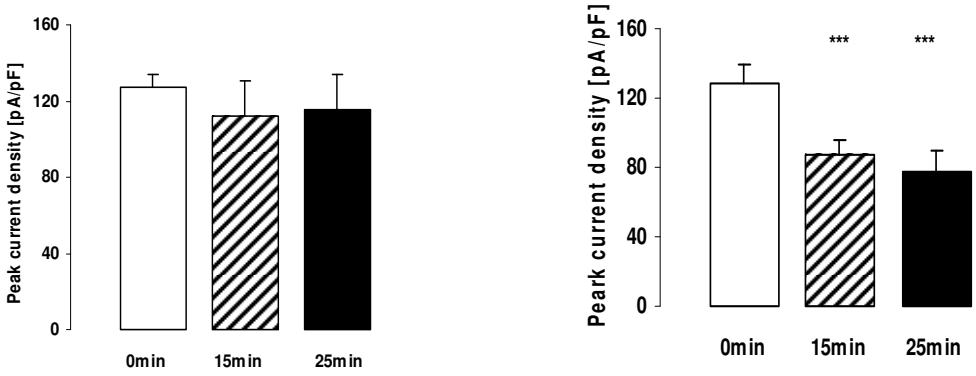


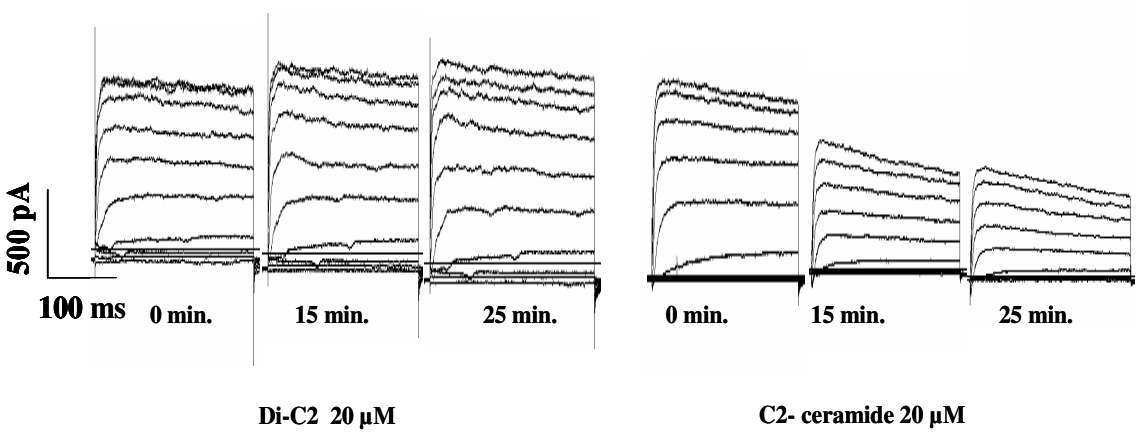
Fig. 23: Effect of IAPP on K_v channel activity in WT mouse islet cells. A: Original tracings of voltage gated K^+ currents prior to, 15 min and 25 min after addition of solvent solution (left measurement) and IAPP (right measurement, 5 µM). Whole cell currents were induced by 200 ms voltage pulses increasing by 20 mV steps from –100 mV to +80 mV. B: I-V relations of mean peak current amplitude normalized to the cell capacitance (mean peak current density, ± SEM, n = 6) prior to (white diamonds), 15 min (white squares) and 25 min (black triangles) after exposure of mouse islet cells to solvent solution without (left measurement) and with (right measurement) 5 µM IAPP. C: Arithmetic means ± SEM (n = 6) of current density at +100 mV prior to (white bars) and following 15 min (hatched bars) and 25 min (black bars) exposure of the cells to solvent (left measurement) or to IAPP (5 µM) (right measurement). *** indicates very significant difference from respective value prior to addition of IAPP (P< 0.001; ANOVA).

4.3.5 Effect of C_2–ceramide on K_v channel activity in WT mouse islet cells

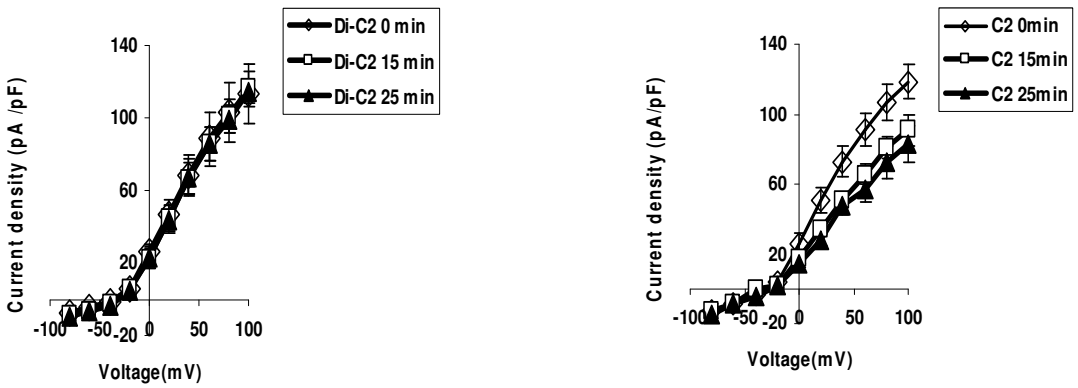
The effect of amyloid peptides (IAPP and amyloid) on whole cell K_v channel current was mimicked by N-acetyl-D-sphingosine (C_2-ceramide, 20 µM) whereas the inactive dihydro-C_2-ceramide (Di-C2, 20 µM) did not significantly modify whole cell outward currents (Fig.24). Di-

C2 failed to reduce K_v channel current after 15 min. and 25 min. (0 min.: 113.33 ± 16.89 pA/pF; 15 min.: 116.20 ± 9.61 pA/pF; 25 min.: 114.17 ± 6.27 pA/pF, n = 5, $P > 0.05$). Application of N-acetyl-D-sphingosine induces the similar effect as IAPP on K_v channel current in WT islet cells (0 min.: 118.33 ± 9.97 pA/pF; 15 min.: 90.92 ± 8.61 pA/pF; 25 min.: 82.61 ± 10.49 pA/pF, n = 6, $P < 0.001$).

A



B



C

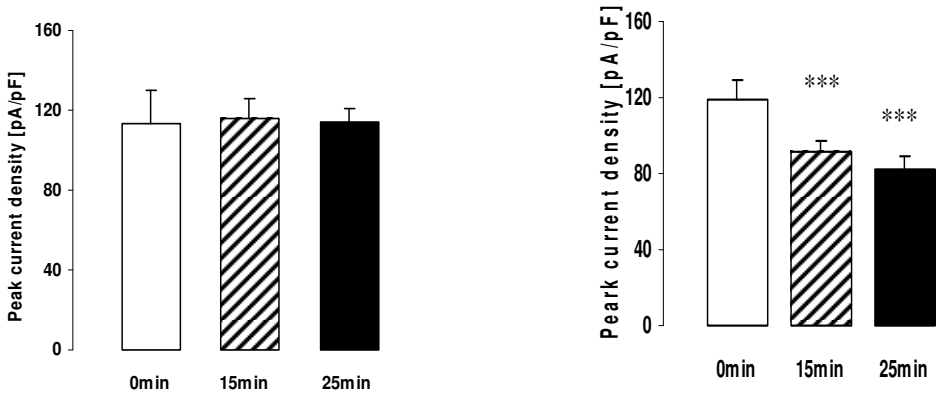


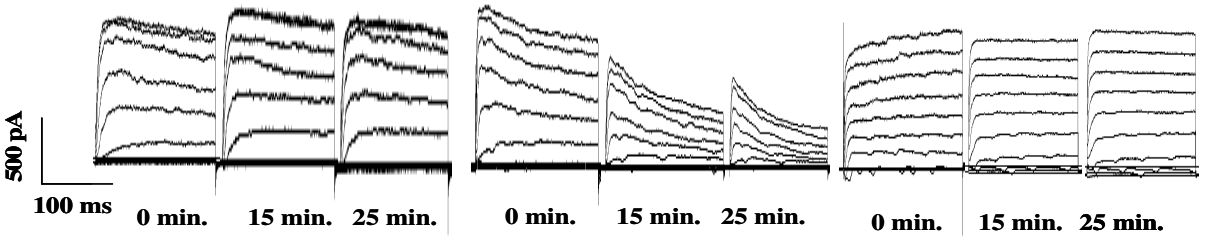
Fig. 24: Effect of C_2–ceramide on K_v channel activity in pancreatic beta cells. A**:** Original tracings of voltage gated K^+ currents prior to, 15 min and 25 min after addition of Di-C_2 (left measurement, 20 µM) and C_2-ceramide (right measurement, 20 µM). B: I-V relations of mean peak current density (± SEM, n = 6 each) prior to (white diamonds), 15 min (white squares) and 25 min (black triangles) exposure to Di-C_2 (left panel) and 20 µM C_2-ceramide (right panel). C: Arithmetic means ± SEM (n = 6) of current density at +100 mV prior to (white bars) and following 15 min (hatched bars) and 25 min (black bars) exposure of the cells in the presence of 20 µM Di-C_2 (left panel) and C_2-ceramide (right panel). *** indicates very significant difference to 0 min. (P< 0.001; ANOVA).

4.3.6 C_2 but not IAPP inhibited K_v channel current in ASM-KO islet cells

As amyloid and ceramide both induce comparable inhibition of K_v channel activities in islet cells, experiments were performed to analyse whether amyloid affects K_v channel activity through ceramide formation. Indeed the application of IAPP to ASM-KO islet cells did not induce any change on K_v channel current (n=6, P> 0.05). In contrast, The administration of C_2-ceramide significantly reduced channel current within 15 min from 119.75 ± 13.29 to 84.49 ± 8.98 (pA/pF, n = 5, P< 0.05) and within 25 min reduced to 62.31 ± 11.92 (n = 5, P< 0.05) (Fig.

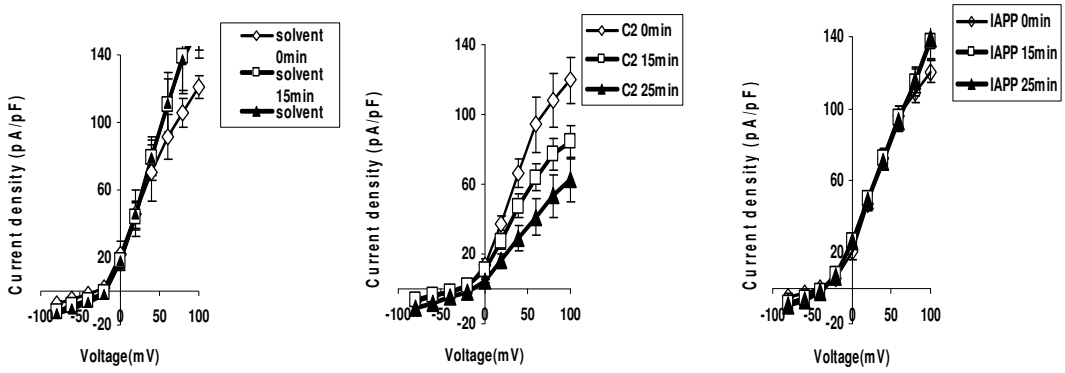
25). These observations suggest the hypothesis that amyloid through the stimulation of ASM and ceramide formation affects ion channel activity in insulin secreting cells.

A



Solvent solution C2 20 µM IAPP 5 µM

B



C

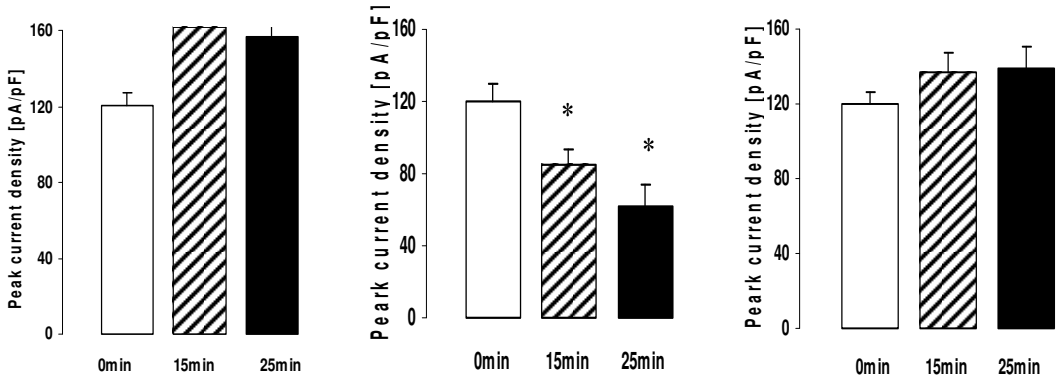


Fig. 25: Effect of IAPP on K_v channel activity in ASM-KO mouse islet cells was abolished. **A:** Original tracings of voltage gated K^+ currents prior to, 15 min and 25 min after addition of solvent solution (left measurement), C_2 20 µM (middle measurement) and IAPP 5 µM (right measurement). Whole cell currents were induced by 200 ms voltage pulses increasing by 20 mV steps from –100 mV to +80 mV. B: I-V relations of mean peak current amplitude normalized to the cell capacitance (mean peak current density, ± SEM, n = 6) prior to (white diamonds), 15 min (white squares) and 25 min (black triangles) after exposure of mouse islet cells to solvent solution (left measurement), C_2 (middle measurement, 20 µM) and IAPP (right measurement, 5 µM). C: Arithmetic means ± SEM (n = 6) of peak current density at +100 mV prior to (white bars) and following 15 min (hatched bars) and 25 min (black bars) exposure of the cells to solvent solution (left measurement), C_2 (middle measurement, 20 µM) and IAPP (right measurement, 5 µM). * indicates significant difference from respective value prior to addition of C_2 ($P< 0.05$; ANOVA).

5 DISCUSSION

The Regulation of membrane ion channel activities plays a crucial role in biological functions in insulin secreting cells. The overwhelming major function of ion channels is the regulation of insulin secretion. But they also influence survival and apoptosis of insulin secreting cells. Insulin is a critically important regulator of whole body energy metabolism. When insulin secretion is absent or reduced, or when peripheral tissues fail to respond to insulin, it results in hyperglycaemia leading ultimately to diabetes. For the understanding of the physiological regulation of insulin secretion and the pathophysiological dysfunction of beta cells the measurement of electrical activity of beta cells and the underlying changes in ion currents was crucial and was made possible due to the introduction of the patch-clamp technique (Hamill et al.1981).

Channel regulators can be roughly divided into two groups: the activators and the inhibitors. Inhibitors of the K_{ATP} , K_V channels close these K^+ channels an effect that depolarizes beta cells stimulate insulin release (Ashcroft et al.1988a; Ashcroft et al.1988b; Rorsman et al. 1990; Chen et al.2007; MacDonald et al.2003b). Physiological inhibitors include nutrients which are metabolized by the beta cell and which generate ATP such as glucose and certain pharmacological agents such as sulfonylureas. Activators of K^+ channels reduce electrical activity of beta cells, induce a hyperpolarization and inhibit insulin release. A number of substances, especially hormones and neurotransmitters that act through Gi coupeled receptors, and pharmacological agents such as diazoxide inhibit electrical activity and insulin secretion. But the way in which they affect channel activities is still not complete clear. The present study explored whether the PI3kinase signalling pathways affects electrical activity of beta cells and whether beta cell ion currents are influenced by substances that affect beta cell survival.

5.1 The Regulation of membrane potential by glucose and tolbutamide

The basic functions and physiology of the beta cells are relatively well understood. The primary function of the beta cell is to correlate the release of insulin with changes in blood glucose concentrations. When blood glucose, an essential nutritional stimulator, increases the

beta cell is stimulated and starts to be electrical active (Henquin et al.1984). Glucose transport protein-2 (GLUT2) and a kinase (glucokinase) act as glucose sensors (Efrat et al.1994; Im et al.2006). These proteins have a low affinity for glucose. Both, the transporter and the enzyme, are halfmaximally active at around 5-5.5 mM (Efrat et al.1994; Im et al.2006; Kuniaki Takata.1996; Watson et al.2001). The presented data show that at a glucose concentration below 5 mM,, beta cells are electrically silent with a resting membrane potential of about - 70 mV. Raising the external glucose concentration to 5.6 mM, due to the low K_m of Glut-2 and of glucokinase, uptake and metabolism is increased. At the same time, membrane potential is elevated to – 60 mV. but no action potentials were generated. Only at glucose concentrations higher than 7 mM, depolarization becomes sufficient to reach the threshold potential (~ - 50 mV) at which electrical activity is initiated and insulin release is stimulated. At higher glucose concentrations 11.2 mM and 16.8 mM the membrane depolarized further and reached the same plateau level (-38.50 ± 1.53 and -38.82 ± 1.07 mV, n= 26 and 38; respectively). Isolated beta cells were continuously active and generated action potentials at glucose concentrations higher that 7 mM. This behaviour is specific for isolated beta cells. Beta cells that remain in the islet and in contact with other islet cells show oscillatory membrane activity at intermediate glucose concentrations. The respone to glucose is slow since the closure of K_{ATP} channels and the depolarisation depends on metabolism and the generation of ATP. Action potentials were generated when glucose was increased from 2.8 mM to 11.2 or 16.8 mM glucose after 66.92 ± 7.37 s and 62.63 ± 6.93 s, respectively. Furthermore, 78.05 % of cells measured responsed to high glucose with the generation of action potentials. This rate is in accordance with the percentage of endocrine cells within the islets immunocytochemically stained for insulin (Elayat et al.1995). These data suggest that mouse beta cells remain responsive to glucose after isolation and cell culture and the glucose repsponsiveness may be used to distinguish beta cells from non beta cells.

Sulphonylureas are a class of anti-diabetic drugs that have been used for T2DM over half of the century. These drugs act as potent and direct blockers of K_{ATP} channel activity by binding to the SUR1 subunit of the K_{ATP} channel. They stimulate insulin release (Boyd. 1988; de Weille et al. 1989; Ashford. 1990). Tolbutamide is the classic example of the first generation of sulphonylureas that consists solely of the sulphonylurea group (Ashford. 1990). The time course for the inhibition of channel activity by tolbutamide is rapid as confirmed in this study. The initiation of action potential is accomplished after 3.6 ± 0.2 s and in 1-2 s the maximal effect was reached. This time course reflects the direct binding of tolbutamide to K_{ATP} channels in contrast

to the slow effect of glucose on membrane potential. The data further show that the inhibition of K_{ATP} current by tolbutamide is reversible. The membrane potential recovered after wash-out of tolbutamide in almost all the measurement (85 out of 93) within 1 min. This may reflect a quick dissociation of the drug-receptor complex. Electrophysiological studies have demonstrated that tolbutamide is specific for K_{ATP} channel as the drug has no significant effect on the K_v current, Ca^{2+} currents, Ca^{2+}activated K^+ channels and non-selective cation channels (Rorsman and Trube. 1986; Trube et al. 1986; Sturgess et al. 1988; Ashcroft et al. 1989a;Gillis et al. 1989). Tolbutamide is therefore a widely used tool to study the role of K^+ channels in insulin secreting cells. In the following experiments tolbutamide was used to block the prominent K_{ATP} currents that allowed the examination of GIRK and Kv channels in isolated mouse beta cells.

5.2 Regulation of membrane potential and membrane currents by leptin, IGF-1, insulin and adrenaline

Leptin, the obesity (ob) gene product, is produced in adipose tissue and regulates the feeding behaviour and energy metabolism. Exogenous leptin has been shown to inhibit glucose-induced insulin secretion of islets isolated from ob/ob and wild-type mice (Emilsson et al. 1997; Keiffer et al. 1997). Electrophysiologiocal studies indicate that in isolated arcuate neurones (Mirshamsi et al. 2004) and in an insulin-secreting cell line CRI-G1 (Harvey et al. 1997), leptin activates K_{ATP} channels and induces a hyperpolarization of insulin secreting cells. The present experiments are in accordance with these findings that leptin activates K_{ATP} channels that leads to a hyperpolarization of pancreatic beta cells and may account for the inhibition of insulin secretion. It has been shown in a haematopoietic cell line that the activation of leptin receptors stimulates janus kinase 2 (JAK2) (Ghilardi& Skoda. 1997). Whether in beta cells the activation of this pathway by leptin underlies its inhibitory effect on secretion is unkown (Ihle.1995). To examine whether the PI3 kinase signalling pathway is involved in leptin mediate effects on membrane potential changes of beta cells LY294002, selective inhibitors of PI 3-kinase (Vlahos et al.1994), was applied. The results demonstrate that LY294002 occluded leptin-induced hyperpolarisation of mouse islet cells and suggest that the effects of leptin on membrane potential involves the activation of IGF-1/insulin/IRS/PI3 kinase signalling (Myers & White, 1996). A

similar observation was made in isolated arcuate neurones, where leptin increased the activity of K_{ATP} channels in a PI3 kinase dependent manner (Mirshamsi *et al*. 2004). Furthermore this study using primary mouse islet cells confirms a similar observation made with an the insulin secreting cell line CRI-G1 cells (Harvey et al.1998). An important lipid that regulates K_{ATP} channel opening is PIP_2 . Treatment of cells with PI3 kinase inhibitors results in the depletion of PIP_2 . The reason for the inhibition of the hyperpolarisation of islet cells by leptin may thus be the depletion of PIP_2. Whether leptin stimulates PIP_2 formation, that leads to the opening of K_{ATP} channels, or whether the effect of leptin is mediated through the induction of actin filament depolymerization as suggested by Mirshamsi et al. needs further analysis (Mirshamsi et al 2004).

Human insulin-like growth factor-I (IGF-1) influences cell growth and cell differentiation and also exerts insulin-like effects on glucose homeostasis. IGF-I infusion *in vivo* decreased plasma levels of lactate (Jacob et al.1989; Jacob et al.1992), amino acids (Jacob et al.1989; Jacob, et al.1992) and glucose (Jacob et al.1989; Zapf et al. 1986). in healthy rats exposed to hyperglycemia. IGF-I infusion dose-dependently reduced plasma insulin and net glucose uptake (Fürnsinn et al.1994). The results demonstrate that IGF-1 is a potent inhibitor of insulin release. The hyperpolarizing effect of IGF-1 on membrane potential shown here may explain the inhibition insulin release induced by IGF-1. The underlying mechanism involves the activation of PI3 kinase as LY29402 inhibited IGF-1 induced hyperpolarisation. Interestingly, mice lacking IGF-1 receptors in beta cells display increased basal (fed and fasted) insulin levels, but defective glucose-stimulated insulin secretion (Kulkarni et al.2002; Xuan et al.2002). These results suggest that IGF-1 receptor signalling that favors beta cell hyperpolarisation might be important for the regulation of basal insulin secretion.

As insulin and IGF-1 activate the same signal transduction pathways, insulin may exert similar effects on beta cell membrane potential as does IGF-1. However the study here shows no significant effect of exogenous insulin on membrane potential in mouse islet cells. This finding confirms a reported study that application of insulin (0.1—1000 nM) had no effect on the resting membrane potential of insulin secreting cells (Harvey et al.1998). Recent data has been shown in mouse islet cells that a 20 min pretreatment with insulin exerted a marked reduction in the sensitivity of beta cell K_{ATP} channels to ATP (Leung et al.2006). This results in a significantly larger outward current in the presence of 1 mM ATP in the pipette solution in cell treated with insulin when compared to untreated cells (Leung et al.2006). Insulin has been postulated to exert a negative-feedback control on its own secretion (Koranyi et al.1992; Marchetti et al.1995).

However, a acute effect of insulin on K_{ATP} channels through the activation of PI3 kinase seems not to be involved.

It is well-known adrenaline acts as antagonist of insulin in that the catecholamine inhibits insulin secretion and enhances blood glucose levels. Adrenline induces a hyperpolarization of insulin secreting cells via a pertussis toxin (PTX)-sensitive mechanism (Abel et al.1996). The effect is mediated through the activation of alpha(2A)- and alpha(2C)-adrenoceptors and the respective intracellular signaling pathways, resulting in an inhibition of adenylylcyclase and a hyperpolarisation (Peterhoff et al.2003). Adrenaline did not influence basal or insulin-stimulated PI 3 kinase activity in rat fast-twitch epitrochlearis muscle (Jensen et al.2008). In contrast activation of alpha1 adrenergic receptors by noradrenaline has been shown to stimulate PI 3-kinase and to increase mitogenesis in human vascular smooth muscle cells (Hu et al.1996). This study analysed whether in insulin secreting cells adrenalin exerts effects dependent on PI-3 kinase signalling pathway. The experiments of this study demonstrate that 1µM adrenaline induced a significant hyperpolarization and activated outward currents in mouse beta cells. Furthermore, the effect of adrenaline was inhibited by both PI 3-kinase inhibitors, LY294002 and wortmannin. These observations suggest that in mouse islet cells, adrenaline inhibits insulin secretion through PI 3-kinase signalling pathway. Adrenaline induced a significant faster hyperpolarisation than leptin or IGF-1. For the understanding of the mechanism of activation by adrenaline further studies are needed. Indeed it is not known whether and by which mechanism adrenaline affects PIP_2.

In conclusion, leptin, IGF-1 and adrenaline induce a hyperpolarization of mouse pancreatic beta cells via a mechanism that involves PI 3-kinase signaling pathway. Exogenous insulin failed to elicit a significant effect on cell membrane potential in mouse beta cells.

5.3 Regulation of amyloid and ceramide on apoptosis and K_V channel activity

Cell death can follow two distinct pathways: apoptosis or necrosis. Morphologically, necrosis is characterized by a dramatic swelling in cell size and rupture of the plasma membrane, with release of the cellular contents into the extracellular space (Gores et al.1990). This consequently results in a damage of neighboring tissues and triggers the production of cytokines

(Haslett.1992). This process has been proved to be a relatively passive process associated with rapid cellular ATP depletion. Unlike necrosis, apoptosis is characterized by cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation and without influence of cytokines (Kerr et al.1965; McConkey et al.1998). Apoptosis describes a active form of cell death and constitutes a mechanism of cell replacement and clearance of damaged cells during several physiological and pathological situations (DeLong. 1998).

In T2DM, the islet typically contains extracellular deposits of amyloid derived from IAPP (Maloy et al.1981; Clark et al.1987; Cooper et al.1987; Johnson et al.1989). Islet amyloid has also been reported in transplanted human islets (Westermark et al.1995; Westermark et al.2005). Thus, amyloid (specially indicates IAPP) deposition became a pathological feature in T2DM that may affect beta cell mass as a result from increased beta cell death.

It has been reported that the amount of islet amyloid rather than the formation islet amyloid deposits is related to increased apoptosis of beta cells in a murine model of T2DM (Butler et al.2003). It is suggested that soluble IAPP oligomers but not islet amyloid are responsible for increased beta cell apoptosis in mice and humans .. On the other hand, other studies showed that amyloid precursor peptides, such as the $Aß_{1-42}$ fragment, triggered suicidal death in a variety of cells including neurons (Abdul et al.2006; Huang and May. 2006; Malaplate-Armand et al.2006; Ran et al.2006; Raynaud and Marcilhac.2006; Yu et al.2006), endothelial cells (Donnini et al.2006), neutrophils (Park et al.2006), erythrocytes (Nicolay et al.2007). This soluble amyloid $Aß_{1-42}$ peptide was used to examine whether soluble amyloid can induce apoptosis in mouse beta cell. The present study reveals that $Aß_{1-42}$ indeed triggers the suicidal death of pancreatic beta cells. This effect of $Aß_{1-42}$ is obviously secondary to stimulation of acid sphiungomyelinase and subsequent formation of ceramide. Accordingly, the effect is mimicked by addition of ceramide. More importantly, genetic knockout of acid sphingomyelinase abrogates the proapoptotic effect of $Aß_{1-42}$. Ceramide has been shown to participate in the triggering of cell death in a variety of cells including T-lymphocytes (Gulbins et al.1997), hepatocytes (Lang et al.2007), erythrocytes (Bentzen et al., 2007; Lang et al., 2006; Nicolay et al., 2006) and pancreatic beta cells (Newsholme et al., 2007).

Recent experiment provides further evidence about the mechanism of apoptotic cell death induced by amyloid. It is shown, that h-IAPP oligomers may act through the interaction with the cell membrane that disturbs cell-to-cell integrity and disrupts the morphology of human islets. These actions of h-IAPP not only induces apoptotic cell death but severely impairs glucose-

mediated insulin secretion (Ritzel et al.2007). Apart from morphological changes, apoptotic pathways induced by amyloids include the ER stress pathway (Haataja et al.2008; Huang et al.2007). Amyloid peptides may be effective through stimulation of Ca^{2+} entry (Park et al.2006), calpain activation (Raynaud and Marcilhac.2006), oxidative stress (Abdul et al.2006; Huang and May. 2006; Ran et al.2006) and ceramide formation (Malaplate-Armand et al.2006).

Here, it is shown that amyloid inhibited K_v channel activity in pancreatic beta cells. Amyloid was again effective through stimulation of ceramide formation. Ceramide has been shown to modify the activity of a variety of ion channels including K^+ channels (Bai et al., 2007; Bock et al., 2003; Chapman et al., 2005; Gulbins et al., 1997; Hida et al., 1998; Ramstrom et al., 2004; Wu et al., 2001; Zhang et al., 2002), Ca^{2+} channels (Chik et al., 2004; Lepple-Wienhues et al., 1999) and Na^+ channels (Liu et al., 2007). In some cells inhibition of K_v channels has been shown to compromise cell proliferation (Patel and Lazdunski.2004; Wang.2004) and facilitate apoptosis (Bankers-Fulbright et al., 1998; Chin et al., 1997; Han et al., 2004; Miki et al., 1997; Pal et al., 2004; Patel and Lazdunski 2004). On the other hand, amyloid (Colom et al., 1998; Yu et al., 1998) and ceramide (Yu et al., 1999) induced apoptotic cell death may be paralleled by activation of K^+ channels. Activation of K^+ channels leads to cell shrinkage, a hallmark of suicidal cell death (Bortner et al., 1997; Bortner and Cidlowski 1999; Bortner and Cidlowski 2004; Maeno et al., 2000; Schneider et al., 2007). This study, however, supports the hypothesis that ceramide does not stimulate but inhibits K_v currents. That K_v channel activity is changed during apoptosis has been shown in Jurkat T lymphocytes, where CD95-induced apoptosis is paralleled by inhibition of $K_v1.3$ channels (Szabo et al. 1996; Szabo et al. 2004). This channel is the cell volume regulatory K^+ channel of Jurkat cells (Deutsch and Chen. 1993). CD95-triggering leads to tyrosine phosphorylation of the $K_v1.3$ channel protein (Gulbins et al.1997; Szabo et al.1996). Accordingly, CD95 induced inhibition of $K_v1.3$ requires the activation of the kinase Lck^{56} (Gulbins et al.1997; Szabo et al.1996). The inhibitory effect of CD95 on Kv1.3 channel activity is mimicked by the sphingomyelinase product ceramide, which similarly induces apoptosis (Gulbins et al.1997; Gulbins et al.2000).

The inhibition of Kv channels in insulijn secreting cells may favour an increased Ca^{2+} load in cells stimulated by hyperglycemia.The notion that a sustained cellular Ca^{2+} overload is highly toxic by causing massive activation of caspases that triggers cell-death signaling pathway and the toxicity of Ca^{2+} ionophores in cultured cells was one of the first mechanisms described (Pressman.1976; Fariss et al.1985). More data, however, have suggested that both at early and

late stages of the apoptotic pathway, the increases of $[Ca^{2+}]c$ can been observed (Kruman et al., 1998; Tombal et al.,1999; Lynch et al., 2000) and both Ca^{2+} release from the ER and capacitative Ca^{2+} influx through Ca^{2+} release-activated Ca^{2+} channels have been proposed to be apoptogenic (Pinton and Rizzuto, 2006). Thus, the concept is that severe Ca^{2+} dysregulation can promote cell death and result in apoptosis. The present experiment of patch clamp reveals, IAPP and ceramide both within 15-25 min decrease K_V channel current in islet cells. The inhibition of Kv channels was only partly reversible after wash-out of amyloid and ceramide. These finding support the isdea that a sustained closure of K_v channels and activation of Ca_v channels results in a long lasting overload of Ca^2. As in ASM-KO islet cells ceramide still induced Kv channel closure and apoptosis while the effect of amyloid was reduced it is concluded that amyloid at least in part induces apoptosis through ASMase and the formation of ceramide. The mechanism underlying amyloid mediated activation of ASM and NSM (neutral sphingomyelinase) has been suggested previously.

In pancreatic beta cells, K_v channels may further participate in the regulation of insulin release (MacDonald et al., 2002c; Ullrich et al., 2005). Accordingly, inhibition of K_v channels augments glucose-induced insulin secretion and increase oxidative stress that may also contribute to the induction of apoptotic cell death (MacDonald et al., 2002c). Conversely, glucocorticoids stimulate $K_v1.5$ channels, thus accelerating the repolarisation of the cell membrane upon glucose induced depolarization and blunting the Ca^{2+} entry through voltage gated Ca^{2+} channels (Ullrich et al., 2005). Increased K_v channel activity may account for reduced insulin secretion observed under glucocorticoid excess.

In conclusion, the study shows for the first time that amyloid as well as ceramide reduce K_v channel activity insulin secreting cells. Further evidences suggest that amyloid triggers apoptosis of pancreatic beta cells at least in part through the activation of ASM and the formation of ceramide. These events may contribute to the pathophysiology of amyloid related diseases such as diabetes mellitus and Alzheimer.

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7. CURRICULUM VITAE

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Berufliche Erfahrungen

2004-2006	**Oberärztin** Abt. Endokrinologie, Universitätsklinik, Guangxi Medizinischen Universität, China.
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1995-2001	**Assistenzärztin** Innere Medizin (Abt. Endokrinologie, Neurologie, Kardiologie, Pneumologie, Hepatologie, Gastroenterologie, Hämatologie, Nephrologie, Gerontologie und internistische Intensivstation), 2. Öffentliches Krankenhaus der Provinz Guangxi, China
1996	**Approbation als Ärztin** V. R. China

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Studium

2007-2009	**Biologie** Universität Tübingen, Deutschland Abschluss: Wissenschaftlicher Doktor im Biologie
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2001-2004	**Innere Medizin** Guangxi Medizinische Universität, V. R. China. Abschluss: Medizinischer Master
1990-1995	**Klinische Medizin** Guangxi Medizinische Universität, V. R. China. Abschluss: Medizinischer Bachelor

Forschungserfahrung

2007-2009	**Doktorarbeit** "Die Rolle und Regulation der Ionkanäle in den Insel-Zellen" Prof. Dr. med. Florian Lang und Prof. Dr. F. Götz, Physiologisches Institut , Universität Tübingen, Deutschland.
2006-2007	**Diplomarbeit** " Dexamethasone stimuliert die Na^+/K^+ ATPase durch die SGK1 in den INS-1 Zellen und B-Zellen." Prof. Dr. med. Florian Lang, Physiologisches Institut , Universität Tübingen, Deutschland.
2005	**Prüfärztin** "Die klinische Studie der Phase IV mit dem Irbesartan bei Patienten mit der Hypertonie " Prof. Dr. med. Zuojie Luo, Abt.Endokrinologie, Guangxi Medizinische Universität, China.

2004-2005	**Prüfärztin** "Die klinische Studie der Phase II mit der Nateglinide bei Patienten mit Typ-2-Diabetes " Prof. Dr. med. Zuojie Luo, Abt.Endokrinologie, Guangxi Medizinische Universität, China.
2003-2004	**Prüfärztin** "Die klinische Studie der Phase II mit der Glipizide und dem Metformin bei Patienten mit Typ-2-Diabetes" Prof. Dr. med. Z. Luo, Abt.Endokrinologie, Guangxi Medizinische Universität, China.
2002-2004	**Die Forschung des Masters** "Die Beziehung zwischen der Resistin, der IL-6 und der Insulinresistenz in der Typ-2-Diabetes" Prof. Dr. med. Z. Luo, Abt. Endokrinologie, Guangxi Medizinische Universität, China.
2002-2004	**Projekt Assistenz** "Die Studie, der Aufbau des Daten-Systems und die Kontrolle der Diabetes am Guangxi" Prof. Dr. med. Q. Chen, Abt. Endokrinologie, Guangxi Medizinische Universität, China.
2002-2003	**Prüfärztin** "Die klinische Studie der Phase II mit Pioglitazone bei Patienten mit Typ-2-Diabetes" Prof. Dr. med. Z. Luo, Abt. Endokrinologie, Guangxi Medizinische Universität, China.

Methodologische Erfahrungen

- Elektrophysiologie: Membranfleckklemme/patch clamp
- Kultivierung primärer und sekundärer Zelllinien
- Immunzytochemie
- Fluoreszenzmikroskopie/konfokale Mikroskopie
- Chirurgische Verfahren an Mäusen
- Western Blot
- Polymerasekettenreaktion und verwandte Techniken
- Mikrobiologische Grundtechniken

Publikationen (peer review)

1 **Zhang Y**, Ranta F, Tang C, Shumilina E, Mahmud H, Fölle M, Ullrich S, Häring HU and Lang F. (2009) Sphingomyelinase dependent apoptosis following treatment of pancreatic beta-cells with amyloid peptides Aß1-42 or IAPP. Apoptosis. 14(7):878-89 .

2 **Ying Zhang**, Ekaterina Shumilina, Hans-Ulrich Häring and Susanne Ullrich,Florian Lang. (2009) Epinephrine-induced hyperpolarization of pancreatic islet cells is sensitive to PI3K-PDK1 signaling. (in press).

3 **Ying Zhang**, Ekaterina Shumilina, Hans-Ulrich Häring and Susanne Ullrich, Florian Lang. (2009) Leptin induced hyperpolarisation of pancreatic beta cells.(Manuscript in preparation).

4 **Zhang, Y**., Shumilina, E., Lang, F., Ullrich, S. (2009) Involvement of phosphoinositide dependent kinase PDK1 in the regulation of channel activity in pancreatic beta cells by epinephrine, Acta Physiologica 195 (Suppl. 669): 121.

5 **Zhang, Y**., Shumilina, E. K., Ullrich, S. and Lang F. (2008) Inhibition of whole cell currents in pancreatic ß cells by ceramide. Acta Physiologica 192 (Suppl. 663): 180.

6 **Zhang, Y.,** Avram, D., Ranta, F., Lang, F. and Ullrich, S. (2007) Regulation of Na+/K+ ATPase by dexamethasone in insulin secreting cells through SGK1. Acta Physiologica 189 (Suppl. 653): 107.

7 Ullrich S, **Zhang Y**, Avram D, Ranta F, Kuhl D, Haring HU, Lang F.(2007) Dexamethasone increases Na^+/K^+ATPase activity in insulin secreting cells through SGK1. Biochem Biophys Res Commun. 19;352(3):662-7.

8 **Ying Zhang**.(2004) Research on resistin and insulin resistance. Foreign Medicine—internal medicine. 31(5):203-205 (review).

9 **Ying Zhang**, Zuijie Luo, Qingyun Chen.(2004) The change of plasma resistin level before and after treatment of piglitazone in patients with T2DM. Journal of Clinical Medicine. 21(7):480-481 .

10 Zuojie Luo, **Ying Zhang**, Xinghuan Liang et al.(2004) Study of resistin and carotid intima-media thickness in patients with T2DM. Journal of Guangxi Medical University. 21(2):160-162.

11 Qiren Huang, **Ying Zhang**.(2002) Application of Naloxine in toxicosis of hypnotic. Chinese Medicine. 15(3):338-339.

12 **Ying Zhang**, Jiande Zhang.(2001) Study of HbA1c and plasma lipid in diabetic patients with or without previous stroke. Clinical Focus. 23(1):21-22.

13 **Ying Zhang**, Jiande Zhang.(2001) Clinical analysis of diabetic patient accompanied with infection. Chinese Medicine.14(1):48-49.

14 **Ying Zhang**, Jiande Zhang, Wenlong Tang.(2001) Analysis of misdiagnosis in senile patients with hypothyroidism. Journal of Guangxi National Medical College of Right River . 23(4):625-626.

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März, 2009	"Involvement of phosphoinositide dependent kinase PDK1 in the regulation of channel activity in pancreatic beta cells by epinephrine." **Y. Zhang**, E. Shumilina, F. Lang, S. Ullrich. Poster präsentation. Deutsche Physiologische Gesellschaft, Jahres-kongress. Giessen, 2009
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März, 2007	"Regulation of Na^+/ K^+ ATPase by dexamethasone in insulin secreting cells through SGK1." **Y. Zhang**, D. Avram , F. Ranta, F. Lang and S. Ullrich. Poster präsentation. Deutsche Physiologische Gesellschaft, Jahreskongress, Hannover, 2007

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