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(Ehem. Leiter: Prof. Dr. M. Rupnik)

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Age-dependent changes in the exocytotic efficacy in Kir6.2 ablated mouse pancreatic β-cells

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Ernest Beaudelaire Tsiaze

aus

Bamunka-Ndop(Kamerun)

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Dekan: Prof. Dr. rer. nat. H. K. Kroemer

I. Berichterstatter: Prof. Dr. med. T. Moser

II. Berichterstatter/in: Prof. Dr. rer. nat. B. Schwappach

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Abbreviations

ATP	Adenosine-triphosphate
$[ATP]_c$	cytosolic concentration of ATP
BIR	β-cells inward rectifiers
$[Ca^{2+}]_c$	cytosolic or intracellular calcium concentration
$_c$AMP	Adenosine 3', 5'cyclic monophosphate
C_m	membrane capacitance
ΔC_m	change in membrane capacitance
$\Sigma\Delta C_m$	cumulative change in membrane capacitance
CNS	central nervous system
C_{slow}	resting membrane capacitance
E	exocytotic efficacy
ECS	extracellular solution
EGTA	Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid
GK	Goto Kakizaki
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HVA	high-voltage activated
ICS	intracellular solution
I_{KATP}	Adenosine-triphosphate -sensitive potassium currents
K_{ATP}	Adenosine-triphosphate sensitive K^+ channel
K_{ca}	Calcium-sensitive voltage-dependent K^+ channels
Kir6.2(6.1)	inward-rectifier potassium channel 6.2 (6.1)
Kv	voltage-dependent K^+ channel
LVA	low-voltage activated
MgADP	magnesium adenosine-diphosphate
NADP	Nicotinamid-adenin-dinukleotid-phosphat
PHHI	hyperinsulinaemic hypoglycaemia of infancy
PKA	Protein kinase A
PKC	Protein kinase C
SUR1(2)	Sulphonylurea receptor 1(2)
uK_{ATP}	ubiquitous K_{ATP}
VACC or VDCC	voltage-activated or voltage dependent calcium channels
ZTE	Zentrale Tierexperimentelle Einrichtung

1 Introduction

1.1 The role of ATP-sensitive potassium channels (K_{ATP} channels)

Endocrine pancreas is a key player in nutrient and glucose homeostasis. The most important hormone that prevents hyperglycaemic excursions is the peptide insulin. The secretion of this hormone from the pancreatic β-cells is being governed by a bulk of mechanism where according to current consensus model the ATP-sensitive K^+ channels (K_{ATP} channels) play an important role *(Ashcroft et al. 1984).*

ATP-sensitive potassium currents (I_{KATP}) were originally discovered in cardiac muscles *(Noma 1983)*. Using patch-clamp technique on cyanide (CN)-treated mammalian heart cells, *Noma* described in *1983*, specific K^+ channels which were depressed by intracellular ATP. These channels, referred to as ATP-sensitive K^+ channels, were suggested to regulate cellular energy metabolism in the control of membrane excitability in cardiac myocytes *(Noma 1983)*. Using the same experimental approach in isolated mouse pancreatic β-cells, it was later demonstrated that glucose modulates electrical activity via metabolically induced changes in the activity of K_{ATP} channels *(Ashcroft et al. 1984).* These channels, later found in various tissues, are involved in a multitude of important physiological processes in which a liaison between electrical activity and cell metabolism is common *(Ashcroft 1988, MacDonald and Wheeler 2003, Seino and Miki 2003).*

K_{ATP} channels thereby contribute to glucose homeostasis by regulating hormone secretion in pancreatic β-cells *(Ashcroft et al. 1984, Koster et al. 2005, Miki et al. 1998).* Besides the regulation of insulin secretion, K_{ATP} channel regulates glucagon secretion from pancreatic α-cells di-

rectly or indirectly by glucose-responsive neurons in the ventromedial hypothalamus during hypoglycaemia and as such mediating the counter-regulatory response to Insulin *(Seino and Miki 2003).* Studies on isolated cells and tissues, and more recently on genetically modified mice revealed also the important function of K_{ATP} channels found in a variety of tissues, including pituitary, muscles and kidney *(Ashcroft 1988, Seino and Miki 2003)*. The involvement of K_{ATP} channels activity in glucose uptake in skeletal muscles as well as in the protection of cardiomyocytes against ischemic injury was suggested in previous experiments on genetically modified mice *(Seino and Miki 2003).* The consensus that K_{ATP} channels couple cell metabolism to membrane excitability is now widely accepted.

1.2 The molecular structure of K_{ATP} channels

In the past decades detail studies on K_{ATP} channels have provided great insight into its molecular structure *(Aguilar-Bryan et al. 1995, Clement et al. 1997, Inagaki et al. 1995, Shyng and Nichols 1997)*. The multitude function of K_{ATP} channels in different organs already suggests the molecular heterogeneity of the channels.

K_{ATP} channels are heteromultimers formed by inward rectifier potassium channel (Kir6.x) and sulphonylurea receptor (SUR) subunits *(Inagaki et al. 1995, Shyng and Nichols 1997)*. Reconstitution of the K_{ATP} channels shows that they are a class of ion channels, requiring both a member of the small inward-rectifier potassium channel family, either Kir6.1 (uK_{ATP}, ubiquitous K_{ATP}) or Kir6.2 (BIR, β-cells inward rectifiers), plus a sulfonylurea receptor, SUR1 or SUR2, which are closely related members of the ATP-binding cassette transporter superfamily *(Aguilar-Bryan et al. 1995, Inagaki et al. 1995).* While the K_{ATP} channels (Kir6.2 +

SUR2A /-B) in cardiac and skeletal muscle membranes are rather similar *(Inagaki et al. 1996)*, there are some few differences between these channels and that found in the β-cells *(Ashcroft 1988, Seino and Miki 2003).*

The pancreatic β-cell K_{ATP} channel is composed of SUR1, the high affinity sulfonylurea receptor and Kir6.2 subunits. SUR1 subunits conglomerate with Kir6.2 subunits in the endoplasmic reticulum and were suggested to chaperon the fully assembled K_{ATP} channels to the plasma membrane forming a hetero-octameric complex with a 4:4 (SUR1-Kir6.2) stoichiometry as shown in *fig. I (Clement et al. 1997, Schwappach et al. 2000, Seino et al. 2000, Shyng and Nichols 1997, Zerangue et al. 1999)*.

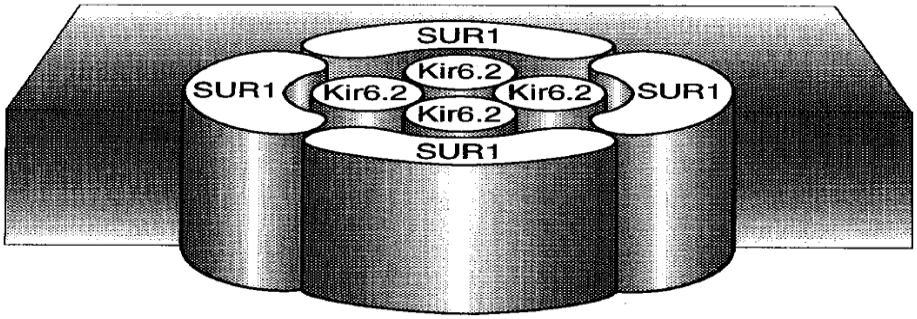

Figure I: Subunit assembly of pancreatic β-cells K_{ATP} channel: The β-cell K_{ATP} channel is a hetero-octamer of Kir6.2 and SUR1 in a 4:4 stoichiometry *(Seino et al. 2000, p. 312).*

1.3 Stimulus-secretion coupling in pancreatic β-cell

Previous excised patch-clamp experiments showed that the activity of the K_{ATP} channels in pancreatic β-cells is regulated by changes in the ATP and ADP concentrations that inhibit or activate K_{ATP} channels, respectively *(Nichols et al. 1996).* SUR1 subunits were reported to confer

activation of the channels by MgADP *(Nichols et al. 1996).* However, more recent whole-cell patch-clamp experiments on pancreas tissue slices could only confirm the ATP-dependent inhibition of K_{ATP} channels, without the effect of ADP *(Speier et al. 2005).* K^+ channel modulators such as stimulatory diazoxide and inhibitory sulfonylureas mainly influence the channels sensitivity to ATP *(Miki et al. 1998, Speier et al. 2005).*

Basically, it appears that the 30-40% increase of ATP above basal level after glucose metabolism is sufficient to initiate the stimulus-secretion coupling cascade *(Maechler et al. 1999).* In addition to forming the K^+ ion-permeable pore, Kir6.2 subunits therefore primarily confer inhibition of the channels by increase in $[ATP]_c$ thereby promoting closure of K_{ATP} channels and inducing membrane depolarization of the β-cells syncytia *(Rupnik 2009, Shyng and Nichols 1997).* Upon depolarization the voltage-dependent calcium channels (VDCC) open, resulting in the elevation of cytosolic calcium and triggering the release of insulin-containing granules *(Ammala et al. 1993)* as shown in *fig. II*.

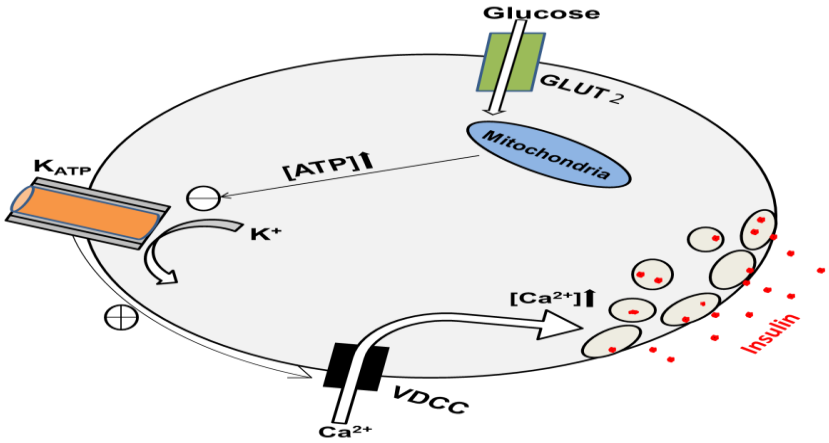

Figure II: The role of pancreatic K_{ATP} channel in insulin secretion: Elevated blood glucose increases glucose metabolism in the β-cells and elevates the cytosolic ATP concentration ($[ATP]_c$). This metabolic signal closes K_{ATP} channels, causing depolarization, activation of voltage-dependent Ca^{2+}- channels (VDCC), Ca^{2+} entry, and insulin exocytosis.

1.4 Defect in stimulus-secretion coupling due to K_{ATP} channel deficiency

So far, we understand that K_{ATP} channel is a delicate participant in the stimulus secretion cascade in pancreatic β-cells *(Ashcroft et al. 1984, Koster et al. 2005, Miki et al. 1998).* A hypo- or hypersecretion of insulin will consequently lead to hyperglycaemia or hypoglycaemia in human with a fatal fate if untreated. In order to maintain normal blood glucose level, a proper functioning K_{ATP} channel is therefore crucial. Accordingly, the subunits of K_{ATP} channel must be fully assembled in order to guarantee the correct function of the channel *(Clement et al. 1997, Schwappach et al. 2000, Shyng and Nichols 1997).* Mutations on any of the subunits protein forming the K_{ATP} channel will impair the functional insulin release and result in an inappropriate serum insulin levels as has been observed in human genetics studies *(Ashcroft and Rorsman 2004).*

Hypoexcitability of the K_{ATP} channel and suppression of insulin release are examined consequences of gain of function mutation of K_{ATP} channels as is the case in neonatal diabetes mellitus (NDM) *(Babenko et al. 2006, Gloyn et al. 2005, Koster et al. 2005).* NDM is characterized by the onset of mild-to-severe hyperglycaemia within the first months of life and could require lifelong therapy. Some cases of permanent or transient neonatal diabetes are caused by mutations in genes encoding the K_{ATP} channel *(Babenko et al. 2006).*

On the other hand, a decrease in blood glucose level implies low glucose metabolic product (low $[ATP]_c$) in pancreatic β-cells and hence activation and opening of the K_{ATP} channels, causing hyperpolarization of the cell membrane and consequent closure of calcium channels. As such, the Influx of calcium is blocked and insulin secretion is suppressed. In this case the blood glucose lowering effect of insulin is

prohibited. A complete loss of K_{ATP} channels, or failure to open, would consequently lead to constitutive insulin secretion and persistent hypoglycaemia which is the case in PHHI patients mostly due to mutations in one or both genes, SUR1 and Kir6.2, encoding the K_{ATP} channel *(Huopio et al. 2002, Lin et al. 2008, Nichols et al. 1996).*

PHHI also referred to as congenital hyperinsulinism is a genetic disorder characterized by deregulated insulin secretion and it is the most common cause of persistent and recurrent hypoglycaemia in infancy *(Aynsley-Green et al, 1981).* It is an autosomal recessive disorder occurring in approximately 1/50,000 births in Western countries and 1/2,500 in some Arabic communities with high rates of consanguinity and is characterized by an excessive release of insulin, despite severe hypoglycaemia *(Aynsley-Green et al. 2000).* These patients commonly exhibit symptomatic hypoglycaemia soon after birth. They are largely unresponsive to K_{ATP} channel openers such as diazoxide and often require subtotal (95%) resection of the pancreas to alleviate hypoglycaemia *(Aynsley-Green et al. 2000)*. Over the past decades rapid advances have been made in understanding the molecular basis of K_{ATP} channel activity and the molecular genetics of PHHI. The majority of causal mutations in PHHI patients that have been uncovered are in the genes encoding the K_{ATP} channel *(Huopio et al. 2002)*.

1.5 Objectives

Parallel to the rapid advances made in understanding the molecular basis of K_{ATP} channel, variable successes were also made in the past in developing animal models mimicking the PHHI disease *(Nichols et al. 1996, Tucker et al. 1997).* Mouse models generated by genetic disruption of the K_{ATP} channels subunits reiterate the expected phenotype that underlies PHHI *(Miki et al. 1998 and 1997, Seghers et al. 2000)*. The genetic disruption of the Kir6.2 subunit (Kir6.2$^{-/-}$) showed a complete K_{ATP} channel-deficiency *(Miki et al. 1998).* This animal model has been used to investigate glucose-induced insulin-secretion in vitro using either physically or enzymatically isolated pancreatic islets or cells *(Miki et al. 1998, Remedi et al. 2006, Seino et al. 2000, Winarto et al. 2001).*

In this study the novel fresh pancreatic slice technique was used to yield information about the systemic compensations related to the channel ablation in the K_{ATP} channel-deficient (Kir6.2 $^{-/-}$) mice. Furthermore, interest was laid on the temporal developments of Islets morphology and glucose homeostasis as well as the stimulus-secretory activities in the Kir6.2$^{-/-}$ mice under this near physiological condition. In addition, this is the first report describing a change in exocytotic efficacy in Kir6.2$^{-/-}$ ablated mice. The results of this study should contribute to our understanding of the pathophysiology of K_{ATP} channel dysfunction in PHHI patients and in animal Models.

2 Material and methods

For detail description of material and methods see publication, Tsiaze et al. 2012.

2.1 Animals

In the past years and decades numerous experimental approaches in studying the role of K_{ATP} channels in various organs were carried on rodents *(Inagaki et al. 1995, Nichols et al. 1996, Seino and Miki 2003, Tucker et al. 1997).* Regarding the K_{ATP} linked β-cells function, genetically disruption of K_{ATP} channel have been performed in the past to generate mice models that reiterates the phenotype of various diseases associated with K_{ATP} channel deficiency *(Aguilar-Bryan et al. 1995, Miki et al. 1997 and 1998, Seghers et al. 2000, Tucker et al. 1997).* As has already been mentioned above, the genetically disruption of the Kir6.2 subunit of the K_{ATP} channel showed a complete K_{ATP} channel deficiency (Kir6.2$^{-/-}$ mice) that portrays the PHHI phenotype *(Miki et al. 1998).* In this study, electrophysiological Experiments were performed on β-cells within intact islets in pancreatic tissue slices obtained from this mice model as stated in *Tsiaze et al. 2012.*

2.2 Pancreatic tissue slice preparation

Over the past decades an immense improvement in β-cells preparation to study insulin secretion has been observed. Back to the 1960s isolated pancreas were used to measure insulin release in rodents *(Sussman et al. 1966)* and later intact islets isolated from pancreatic tissue by micro-dissection were used to study electrical activity (Dean and Matthews 1968). Over time, more in vitro methods such as enzy-

matic isolation of islets *(Lacy and Kostianovsk 1967)* and dispersed islets cell cultures (Lernmark 1974) were developed.

Basically, a complete in vivo method implies conducting experiments on intact animals. The techniques of pancreas and islets isolation were pretty close to in vivo conditions but the complexity of the organ rendered experimental studies at cellular and ionic level difficult. Upon development of the patch clamp technique which enables the study of ionic components of small cells such as β-cells, much has been done in the following decades in the improvement of β-cells preparation. In various organs tissue slices have been used as an *in vitro* system to study organ function close to *in vivo* conditions. The pancreatic tissue slice technique solves the problem of organ complexity and retains the integrity of the tissue *(Speier and Rupnik 2003)*. Additionally, the retained cell-to-cell contacts and intracellular communication are, for example, vital in the study of electrical activities in pancreatic β-cells *(Speier and Rupnik 2003, Speier et al. 2007)*. In this study the pancreatic tissue slice technique was used as briefly described in *Tsiaze et al. 2012*. Embedding the pancreas in agarose did not only provide sufficient mechanical support for efficient electrophysiological experiments on the deeper cell layers of the islets of Langerhans (higher presence of β-cells) and enabled prolonged patch-clamp recordings *(Speier and Rupnik 2003)* but also help distinguish the soft mimicry pancreatic tissue from the mesenteric adipose tissue to which it is tightly associated *(fig. III, left)*. Automated slicer such as vibratom made it possible to produce tissue slices in a rapid and reproducible way *(fig. III, right).*

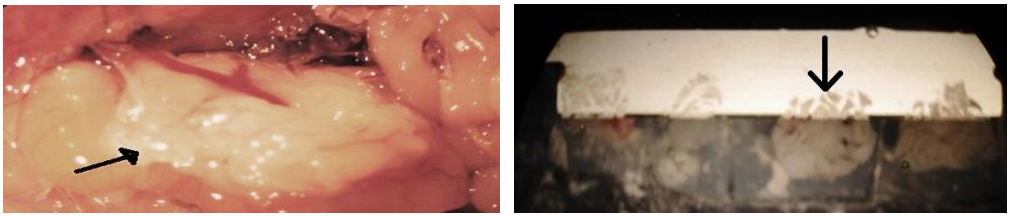

Figure III: **Left**: Agarose-embeded pancreas in situ (black arrow). **Right**: Acute pancreatic tissue slices in the cutting process on a vibratom (black arrow).

2.3 Electrophysiology

In 1968 the first evidence for glucose-stimulated electrical activity in β-cells was demonstrated *(Dean and Matthews, 1968*). The coupling of membrane excitability with exocytosis could be verified using the patch clamp technique that allows the study of individual ion channels in cells as well as the high resolution measurements of small changes in membrane size that occurs during exocytosis in the process of hormone secretion *(Neher and Marty, 1982).* In classical patch clamp technique, the electrode used is a glass pipette. The patch clamp set-up for electrophysiological experiments was prepared as described in *Tsiaze et al. 2012.*

3 Results and discussion

For detail description of results and representative figures see publication, Tsiaze et al. 2012.

3.1 K_{ATP} channels are vital for β-cells survival and tissue architecture

The genetic basis of PHHI is heterogeneous. Most mutations identified up till date reside in the SUR1 subunit. Relatively few mutations have been reported in the Kir6.2 subunit *(Huopio et al. 2002, Lin et al. 2008).* Nevertheless, the homozygous Kir6.2$^{-/-}$ mice have been proved to lack K_{ATP} channel activity and could serve as a PHHI model *(Miki et al. 1998).* The pancreatic tissue slice technique provided a solid background for visualizing the cyto-architecture of the pancreas in its near physiological milieu.

However, the observation of fresh pancreatic slices under transmitted light microscope revealed some age-dependent morphological variations of the islets of Langerhans *(fig 1(a), Tsiaze et al. 2012, p. 53).* While the compacted nature of β-cells arrangement in Kir6.2$^{-/-}$ mice younger than 4 weeks were rather similar to control C57/B6 islets, the aged Kir6.2$^{-/-}$ mice showed a less compacted β-cells arrangement with hollow empty spaces *(fig. 1(a) bottom right, Tsiaze et al. 2012, p. 53).* These apparent differences may reflect the impact of K_{ATP} channels in the late morphological development of islets and insulin-secreting β-cells. Previous studies that showed decrease in number of β-cells from the age of 40 weeks attributed the time course β-cells death to calcium toxicity resulting from measured high basal calcium in β-cells from Kir6.2$^{-/-}$ mice *(Winarto et al. 2001).* However, in this study, the morphological changes of the islets appeared to start earlier than 40 weeks. It is unclear if the more physiological milieu of the fresh islet slice prepa-

ration plays a role. A direct measure of the $[Ca^{2+}]_c$ under this near physiological condition may further clarify this in the future.

3.2 Relationship between islets morphology and glucose metabolism in Kir6.2$^{-/-}$ mice

The aforesaid earlier onset morphological changes in kir6.2$^{-/-}$ mice may reflect the defective glucose metabolism in these animals. A simple random measurement of the blood glucose level revealed also an age dependent metabolic variations in Kir6.2$^{-/-}$ mice. While the young (2 – 4 weeks old) Kir6.2$^{-/-}$ mice were hypoglycaemic, normoglycaemia was measured in young adult mice (5 – 28 weeks old), and a mild hyperglycaemia in aged adult mice (30 – 60 weeks old) *(fig 1(b), Tsiaze et al. 2012, p. 53).* The previous mentioned progressive decreasing population of β-cells in the ageing Kir6.2$^{-/-}$ mice may explain this *(Miki et al. 1998, Remedi et al. 2006, Seino et al. 2000).* Note that the non-nutrient stimulus-secretion of β-cells from adult or aged Kir6.2$^{-/-}$ mice in this study did not really differ to age-matched control *(fig. 4(b) 3 and 4, Tsiaze et al. 2012, p. 57).* This implies that the stimulus-secretion mechanism in ageing kir6.2$^{-/-}$ β-cells may not be that perturbed as was expected. An overall reduction in insulin production due to the reduced number of β-cells in the ageing Kir6.2$^{-/-}$mice may explain the hyperglycaemic metabolic state in the adults. Similar as reported previously the young Kir6.2$^{-/-}$ mice developed normally and there were no apparent abnormalities in general appearance and behaviour compared to age-matched control despite of the transient hypoglycaemia. On the contrary the colony of aged Kir6.2$^{-/-}$ mice also became obese as previously observed *(Kanezaki et al. 2004).*

3.3 Electrophysiological characterization of β-cells in pancreatic tissue slices

In order to study the electrophysiological properties of β-cells in the native islets of fresh pancreatic tissue slice, the identification of the β-cells is crucial. β-cells were identified as described in *fig. 2, Tsiaze et al. 2012, p. 54.* Mouse β-cells react to stimulatory glucose concentrations with a characteristic bursting pattern of electrical activity *(fig. 2(b), Tsiaze et al. 2012, p. 54).* Human as well as rodent β-cells express Connexin36 (Cx36) proteins that form low conducting, weak voltage dependent gap junctions between neighboring cells *(Serre-Beinier et al. 2000).* The resulting electrical coupling is believed to synchronize the secretory activity of β-cells, decrease cell to cell heterogeneity and by that generate an "excitable syncytium" *(Speier et al. 2007).* In the acute tissue slice preparations, this physiological component of the islet is well preserved *(Speier and Rupnik, 2003).*

The displayed electrical activity already at non-stimulatory glucose concentration (3 mM) observed in Kir6.2$^{-/-}$ β-cells *(fig 2(b) bottom panel, Tsiaze et al. 2012, p. 54)* reiterates the persistent depolarized membrane of the Kir6.2$^{-/-}$β-cells. Furthermore, the bursting and spiking activity as well as presence of action potentials during the interburst periods in Kir6.2$^{-/-}$ β-cells *(fig. 2(b) bottom panel inset, Tsiaze et al. 2012, p. 54)* might be an expression of a complete or partial K_{ATP} channel-independent action potential modulated by other channels or metabolic agents. Modulation of VDCC by voltage-dependent K^+ (Kv) and Ca^{2+}-sensitive voltage-dependent K^+ (K_{Ca}) channels were recently described *(MacDonald and Wheeler 2003).* Inhibition of these channels will prolong the action potential and therefore increase the activity of VDCC and thus insulin secretion. The metabolic generation of NADPH contributes to β-cells electrical excitability in response to glucose by reduc-

ing the effective ability of Kv currents to hyperpolarise the cell membrane *(MacDonald and Wheeler 2003).*

3.4 Stimulus-secretion coupling in β-cells of Kir6.2$^{-/-}$ and control C57/B6 mice

However, it is well established that the stimulus-secretory assay in pancreatic β-cells is mainly governed by K_{ATP} channels that primarily function as ATP sensors in the regulation of cellular energy *(Speier et al. 2005).* The above mentioned electrical activity subsequently reflects an increase in $[Ca^{2+}]_c$ that should cause exocytosis of insulin-containing granules and insulin secretion *(Ammala et al. 1993, MacDonald and Wheeler 2003).* The fusion of insulin granules with the cell-membrane leads to increase in membrane surface area. Considering the fact that this change in membrane surface area is proportional to the change in its electrical capacitance, high resolution measurement of the membrane capacitance (C_m) therefore enables an indirect measure of the exocytotic activity *(Neher and Marty, 1982).* Using the patch clamp technique this high resolution measurement of capacitance change could be realized in this study as previously described *(Rose et al. 2007, Sedej et al. 2005, Speier and Rupnik 2003)*.

Capacitance measurements have repeatedly been used as a high resolution assay to asses exocytosis involve in hormone secretion *(Barg et al. 2001, Rose et al. 2007, Sedej et al. 2005, Skelin and Rupnik 2011, Speier and Rupnik 2003).* The glucose-induced stimulus coupling is known to be defective in Kir6.2$^{-/-}$ β-cells *(Miki et al. 1998).* Previous experiments carried out on this animal model were mostly focussed on the blood glucose, basal insulin and calcium measurements in physically or enzymatically isolated islets and incubation experiments *(Miki*

et al. 1998, Seino et al. 2000, Winarto et al. 2001). This report is the first attempt to assess the impact of complete lack of the K_{ATP} channels in this physiological process using acute pancreatic slice preparation. The observed metabolic and morphological changes as well as previously mentioned high basal calcium levels in the K_{ATP} ablated mice suggest a substantial impairment in the late step of the stimulus-secretion cascade. In order to check how this modified secretory machinery in β-cells from Kir6.2$^{-/-}$ mice may modify the glycaemia status, detail examination of the stimulus-secretion coupling in both Kir6.2$^{-/-}$ and control C57/B6 β-cells were performed. In whole-cell voltage-clamp mode depolarization protocol *(fig. 4(b)1 upper trace, Tsiaze et al. 2012, p. 57)* was used to carry out capacitance measurements and as such assess the size of the pool of the vesicles that participated in the exocytotic activity and hence insulin-secretion in both phenotypes.

Referring to the histograms in *fig. 4(a), Tsiaze et al. 2012, p.57* two subpopulations of β-cells could be demonstrated in Kir6.2$^{-/-}$ mice. Retrograde analysis showed that the smaller subpopulation with the excessive C_m change in response to train of depolarization pulses belonged exclusively to Kir6.2$^{-/-}$ mice younger than 4 weeks which were hypoglycaemic *(fig. IV B).* Contrarily, β-cells from Kir6.2$^{-/-}$ mice older than 4 weeks showed surprisingly C_m changes comparable to age matched control *(fig. 4(b) 3 and 4, Tsiaze et al. 2012, p. 57)* but in general, they secreted less than the young kir6.2$^{-/-}$ mice and were normo- to hyperglycaemic *(fig. IV B).* The excessive C_m response in the young kir6.2$^{-/-}$ mice may be due to several reasons which are going to be elaborated in the last two topics.

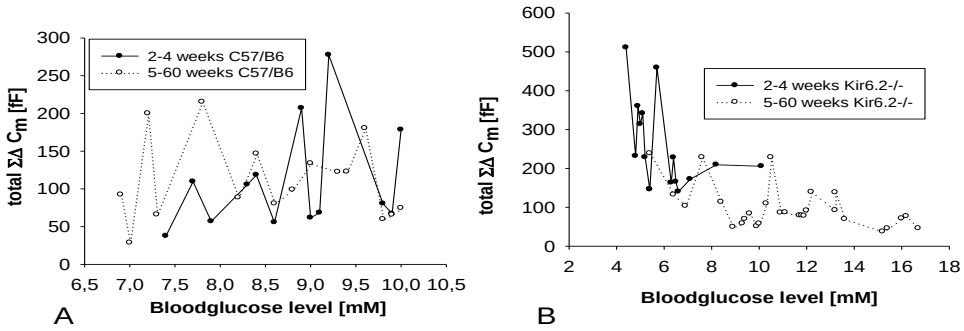

Figure IV: Total membrane capacitance change after train of 50 depolarization pulses of β-cells from individual control C57/B6 (A) and Kir6.2$^{-/-}$ (B) mice versus their respective blood glucose levels. Note the excessive secretion with subsequent mild hypoglycemia in 2-4 weeks Kir6.2$^{-/-}$ mice (B black line with closed circles)

3.5 Ca^{2+} currents flow through voltage dependent calcium channels (VDCC)

In this study, significant larger HVA/LVA peak currents were observed in Kir6.2$^{-/-}$ compared to control C57/B6 *(fig. 3(b), Tsiaze et al. 2012, p. 55).* These larger HVA currents imply high Ca^{2+}charge entry and subsequent rise in $[Ca^{2+}]_c$ which may trigger the excessive secretion observed in the Kir6.2$^{-/-}$ β-cells. The large HVA currents in Kir6.2$^{-/-}$ β-cells may also explain the elevated basal $[Ca^{2+}]_c$ measured in previous experiments *(Seino et al. 2000).*

3.6 Prominent Ca^{2+} secretion coupling in Kir6.2$^{-/-}$ β-cells after two successive trains of depolarization

Furthermore, a prominent Ca^{2+}-dependent exocytotic activity was observed in β-cells from Kir6.2$^{-/-}$ mice upon application of a second train of depolarization 4 min after the first train *(fig. 5(a) 2 and 3, Tsiaze et al. 2012, p. 58).* This augmentation didn't take place in control C57/B6 (*fig. 5(a) 1 and 3, Tsiaze et al. 2012, p. 58).* To further check if this activity-dependent augmentation of C_m is due to calcium sensitivity, the efficacy of calcium to trigger secretion was analysed. A higher Ca^{2+} efficacy was observed in Kir6.2$^{-/-}$ β-cells *(fig. 5(b) 2 and 3, Tsiaze et al. 2012, p. 58)* after the second train of depolarization compared to control C57/B6 β-cells *(fig. 5(b) 1 and 3, Tsiaze et al. 2012, p.58).* This implies that repetitive trains of depolarizing pulses led to augmented efficacy of calcium to trigger secretion in Kir6.2$^{-/-}$ β-cells. This suggests an increase sensitivity of residual calcium ions after the 1st train of depolarisation ready to facilitate the exocytotic machinery. The exact mechanism of this facilitation is yet unknown. The studies on GK rat β-cells suggested persistent increase in $[Ca^{2+}]_c$ that triggers PKC (protein kinase C) activity causing increase in the size or Ca^{2+} sensitivity of the RP (releasable pools) or LDCV (large dense core vesicles) *(Rose et al. 2007).* This was a non-nutrient activity-dependent hypersecretion that was not seen in glucose-modulated stimulation *(Rose et al. 2007).* Further studies on this nonnutrient pathway may provide more insight in developing therapeutic drugs in cases of PHHI attributed to K_{ATP} channel-deficiency.

4 Summary

The tissue slice technique provides a solid field of work for electrophysiological experiments. This novel approach enabled the reassessment of morphology and electrophysiological properties of Kir6.2$^{-/-}$ β-cells in this study. The observed age-linked differences in cyto-architecture reflect a reduced number of β-cells in ageing Kir6.2$^{-/-}$ mice. Moreover, the transient hypoglycaemia in young and a progressive hyperglycaemia in ageing Kir6.2$^{-/-}$ mice could partially be linked to the aforementioned morphological changes. The stimulus-secretory cascade underlying insulin secretion is defective in K_{ATP} ablated Kir6.2$^{-/-}$ mice. The constitutive bursting and spiking electrical activity may explain the large HVA/LVA currents observed in pancreatic β-cells from Kir6.2 $^{-/-}$ mice. This implies higher Ca^{2+} charge entry which may equate to an elevated basal calcium concentration. This large HVA/LVA Ca^{2+}current size and density in Kir6.2$^{-/-}$ mice provided more insight in clarifying the late steps of the metabolic-excitation coupling. Membrane capacitance measurements revealed an excessive C_m response in the young than in ageing Kir6.2$^{-/-}$ mice. This activity-dependent augmentation of C_m was further verified by the application of repetitive trains of stimulation. A prominent exocytosis was observed in β-cells from Kir6.2 $^{-/-}$ mice after second train of depolarization. Moreover, the efficacy of Ca^{2+} to trigger secretion was also increased in Kir6.2$^{-/-}$ β-cells after second train of depolarization. This activity dependent augmentation didn't take place in control C57/B6. This suggests an increase sensitivity of residual calcium ions after the 1st train of depolarisation ready to facilitate the exocytotic machinery in Kir6.2$^{-/-}$ mice. These findings should provide more insight in understanding the pathophysiology of PHHI and other K_{ATP} related diseases.

5 Literature

Aguilar-Bryan L, Nichols CG, Wechsler SW, Clement JP 4th, Boyd AE 3rd, González G, Herrera-Sosa H, Nguy K, Bryan J, Nelson DA (1995): Cloning of the beta cell high-affinity sulfonylurea receptor: a regulator of insulin secretion. Science 268, 423-426

Ammala C, Eliasson L, Bokvist K, Larsson O, Ashcroft FM, Rorsman P (1993): Exocytosis elicited by action potentials and voltage-clamp calcium currents in individual mouse pancreatic B-cells. J Physiol 472, 665-688

Ashcroft FM (1988): Adenosine 5 -triphosphate-sensitive potassium channels. Ann Rev Neurosci 11, 97-118

Ashcroft FM, Rorsman P (2004): Molecular defects in insulin secretion in type-2 diabetes. Rev Endocr Metab Disord 5, 135-142

Ashcroft FM, Harrison DE, Ashcroft SJH (1984): Glucose induces closure of single potassium channels in isolated rat pancreatic β-cells. Nature 312, 446-448.

Aynsley-Green A, Hussain K, Hall J, Saudubray JM, Nihoul-Fékété C, De Lonlay-Debeney P, Brunelle F, Otonkoski T, Thornton P, Lindley KJ (2000): Practical management of hyperinsulinism in infancy. Arch Dis Child Fetal Neonatal Ed 82, F98-F107

Aynsley-Green A, Polak JM, Bloom SR, Gough MH, Keeling J, Ashcroft SJ, Turner RC, Baum JD (1981): Nesidioblastosis of the pancreas: definition of the syndrome and the management of the severe neonatal hyperinsulinaemic hypoglycaemia. Arch Dis Child 56, 496-508

Babenko AP, Polak M, Cavé H, Busiah K, Czernichow P, Scharfmann R, Bryan J, Aguilar-Bryan L, Vaxillaire M, Froguel P (2006): Activating mutations in the ABCC8 gene in neonatal diabetes mellitus. N Engl J Med 355, 456-466

Barg S, Ma X, Eliasson L, Galvanovskis J, Gopel SO, Obermuller S, Platzer J, Renstrom E, Trus M, Atlas D, Striessnig J, Rorsman P (2001): Fast exocytosis with few Ca(2+) channels in insulin secreting mouse pancreatic B cells. Biophys J 81(6), 3308-23

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

Dean PM, Matthews EK (1968): Electrical activity in pancreatic islet cells. Nature 219, 389-390

Gloyn AL, Reimann F, Girard C, Edghill EL, Proks P, Pearson ER, Temple IK, Mackay DJ, Shield JP, Freedenberg D, Noyes K, Ellard S, Ashcroft FM, Gribble FM, Hattersley AT (2005): Relapsing diabetes can result from moderately activating mutations in KCNJ11. Hum Mol Genet 14, 925-934

Huang YC, Rupnik M, Gaisano HY (2011): Unperturbed islet alpha-cell function examined in mouse pancreas tissue slices. J Physiol 589, 395-408

Huopio H, Shyng SL, Otonkoski T, Nichols CG (2002): K(ATP) channels and insulin secretion disorders. Am J Physiol Endocrinol Metab 283, E207-216

Inagaki N, Gonoi T, Clement JP, Compton RN, Reinhardt PW, Cooper CD, Noriyuki JN, Inazawa J, Gonzalez G, Aguilar-Bryan L, Seino S, Bryan J (1995): Reconstitution of IKATP: an inward rectifier subunit plus the sulfonylurea receptor. Science 270, 1166-1170

Inagaki N, Gonoi T, Clement JP, Wang CZ, Aguilar-Bryan L, Bryan J, Seino S (1996): A family of sulfonylurea receptors determines the pharmacological properties of ATP-sensitive K^+ channels. Neuron 16, 1011-1017

Kanezaki Y, Obata T, Matsushima R, Minami A, Yuasa T, Kishi K, Bando Y, Uehara H, Izumi K, Mitani T, Matsumoto M, Takeshita Y, Nakaya Y, Matsumoto T, Ebina Y (2004): K(ATP) channel knockout mice crossbred with transgenic mice expressing a dominant-negative form of human insulin receptor have glucose intolerance but not diabetes. Endocr J 51, 133-144

Koster JC, Permutt MA, Nichols CG (2005): Diabetes and insulin secretion: the ATP-sensitive K^+ channel (K ATP) connection. Diabetes 54, 3065-3072

Lacy PE and Kostianovsky M (1967): Method for the isolation of intact islets of Langerhans from the rat pancreas. Diabetes 16(1), 35-9

Lernmark A (1974): The preparation of, and studies on, free cell suspensions from mouse pancreatic islets. Diabetologia 10(5), 431-8

Lin YW, Bushman JD, Yan FF, Haidar S, MacMullen C, Ganguly A, Stanley CA, Shyng SL (2008): Destabilization of ATP-sensitive potassium channel activity by novel KCNJ11 mutations identified in congenital hyperinsulinism. J Biol Chem 283, 9146-9156

MacDonald PE, Wheeler MB (2003): Voltage-dependent K+ channels in pancreatic beta cells: Role, regulation and potential as therapeutic targets. Diabetologia 46, 1046–1062

MacDonald PE, Obermuller S, Vikman J, Galvanovskis J, Rorsman P, Eliasson L (2005): Regulated exocytosis and kissandrun of synaptic like microvesicles in INS1 and primary rat β-cell. Diabetes 54(3), 736–43.

Maechler P, Kennedy ED, Sebo E, Valeva A, Pozzan T, Wollheim CB (1999): Secretagogues modulate the calcium concentration in the endoplasmic reticulum of insulin-secreting cells. Studies in aequorin-expressing intact and permeabilized ins-1 cells. J Biol Chem 274, 12583-12592

Miki T, Nagashima K, Tashiro F, Kotake K, Yoshitomi H, Tamamoto A, Gonoi T, Iwanaga T, Miyazaki J, Seino S (1998): Defective insulin secretion and enhanced insulin action in KATP channel-deficient mice. Proc Natl Acad Sci U S A 95, 10402-10406

Miki T, Tashiro F, Iwanaga T, Nagashima K, Yoshitomi H, Aihara H, Nitta Y, Gonoi T, Inagaki N, Miyazaki J, Seino S (1997): Abnormalities of pancreatic islets by targeted expression of a dominant-negative KATP channel. Proc Natl Acad Sci USA 94, 11969-11973

Neher E, Marty A (1982): Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells. Proc Natl Acad Sci U S A 79(21), 6712-6

Nichols CG, Shyng SL, Nestorowicz A, Glaser B, Clement JP 4th, Gonzalez G, Aguilar-Bryan L, Permutt MA, Bryan J (1996): Adenosine diphosphate as an intracellular regulator of insulin secretion. Science 272, 1785-1787

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

Remedi MS, Rocheleau JV, Tong A, Patton BL, McDaniel ML, Piston DW, Koster JC, Nichols CG (2006): Hyperinsulinism in mice with heterozygous loss of K(ATP) channels. Diabetologia 49, 2368-2378

Rose T, Efendic S, Rupnik M (2007): Ca^{2+}-secretion coupling is impaired in diabetic Goto Kakizaki rats. J Gen Physiol 129, 493-508

Rupnik M (2009): All together now: exocytose or fail. Islets 1(1), 78-80

Schwappach B, Zerangue N, Jan YN, Jan LY (2000): Molecular basis for K(ATP) assembly: transmembrane interactions mediate association of a K+ channel with an ABC transporter. Neuron 26, 155-167

Sedej S, Rose T, Rupnik M (2005): cAMP increases Ca2+-dependent exocytosis through both PKA and Epac2 in mouse melanotrophs from pituitary tissue slices. J Physiol 567, 799-813

Seghers V, Nakazaki M, DeMayo F, Aguilar-Bryan L, Bryan J (2000): Sur1 knockout mice. A model for K(ATP) channelindependent regulation of insulin secretion. J Biol Chem 275, 9270-9277

Seino S, Iwanaga T, Nagashima K, Miki T (2000): Diverse roles of K(ATP) channels learned from Kir6.2 genetically engineered mice. Diabetes 49, 311-318

Seino S, Miki T (2003): Gene targeting approach to clarification of ion channel function: studies of Kir6.x null mice. J Physiol 554, 295-300

Serre-Beinier V, Le Gurun S, Belluardo N, Trovato-Salinaro A, Charollais A, Haefliger JA, Condorelli DF, Meda P (2000): Cx36 preferentially connects β-cells within pancreatic islets. Diabetes 49, 727-734

Shyng S, Nichols CG (1997): Octameric stoichiometry of the KATP channel complex. J Gen Physiol 110, 655-664

Skelin M, Rupnik M (2011): cAMP increases the sensitivity of exocytosis to Ca^{2}+ primarily through protein kinase A in mouse pancreatic beta cells. Cell calcium 49, 89-99

Speier S, Rupnik M (2003): A novel approach to in situ characterization of pancreatic beta-cells. Pflugers Archiv 446, 553-558

Speier S, Yang SB, Sroka K, Rose T, Rupnik M (2005): KATP-channels in beta-cells in tissue slices are directly modulated by millimolar ATP. Mol Cell Endocrinol 230, 51-58

Speier S, Gjinovci A, Charollais A, Meda P, Rupnik M (2007): Cx36-mediated coupling reduces beta-cell heterogeneity, confines the stimulating glucose concentration range, and affects insulin release kinetics. Diabetes 56, 1078-1086

Sussman KE, Vaughan GD, Timmer RF (1966): An in vitro method for studying insulin secretion in the perfused isolated rat pancreas. Metabolism 15, 466-476

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

Winarto A, Miki T, Seino S, Iwanaga T (2001): Morphological changes in pancreatic islets of KATP channel-deficient mice: the involvement of KATP channels in the survival of insulin cells and the maintenance of islet architecture. Arch Histol Cytol 64, 59-67

Zerangue N, Schwappach B, Jan YN, Jan LY (1999): A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K(ATP) channels. Neuron 22, 537-548

6 Publication

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Age-dependent changes in the exocytotic efficacy in Kir6.2 ablated mouse pancreatic *β*-cells

Ernest Beaudelaire Tsiaze[1], Ya-Chi Huang[2], Lidija Križančić Bombek[3], Shi-Bing Yang[4], Marko Jevšek[3], Susumu Seino[5], Marjan Slak Rupnik[1,3*]

[1]European Neuroscience Institute Göttingen, Germany
[2]Institute of Medical Science, University of Toronto, Toronto, Canada
[3]Institute of Physiology, Faculty of Medicine, University of Maribor, Maribor, Slovenia
[4]Howard Hughes Medical Institute, San Francisco, USA
[5]Division of Diabetes and Endocrinology, Department of Internal Medicine, Kobe University Graduate School of Medicine, Kobe Japan
Email: *marjan.rupnik@uni-mb.si

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ABSTRACT

In this study, we aimed to examine the electrophysiological properties of *β*-cells in Kir6.2$^{-/-}$ mice using fresh pancreatic tissue slice preparation. This preparation is advantageous since it preserves socio-cellular context of the *β*-cells. Using this novel approach we revisited basic morphology and used whole-cell patch-clamp to study electrical excitability as well as to assess the modulation of the late steps of the exocytotic activity of *β*-cells by cytosolic [Ca^{2+}] changes in control and Kir6.2$^{-/-}$ mice. We found that young Kir6.2$^{-/-}$ mice (2 - 4 weeks old) were hypoglycaemic while aged Kir6.2$^{-/-}$ mice (5 - 60 weeks old) were normo- or even hyperglycaemic. Membrane capacitance measurements showed more efficient Ca^{2+}-secretion coupling in young Kir6.2$^{-/-}$ mice, but this coupling is significantly reduced in older Kir6.2$^{-/-}$ mice. We have found increased exocytotic efficacy induced by repetitive trains of depolarization pulses which may result from higher cytosolic [Ca^{2+}] due to hyperexcitability in Kir6.2$^{-/-}$ mice. This condition in turn resulted in the reduced *β*-cell number and function in the following weeks. Detailed assessment of the efficacy of Ca^{2+} dependent exocytosis in *β*-cell from Kir6.2$^{-/-}$ mice may contribute to our understanding of the pathophysiology of persistent hyperinsulinemia hypoglycemia of infancy (PHHI) and suggest potential alternative therapeutic approaches for PHHI patients.

Keywords: Islets of Langerhans; Insulin; Hyperinsulinism; Persistent Hyperinsulinemia Hypoglycemia of Infancy; Ion Channels; Patch-Clamp Techniques

*Corresponding author.

1. INTRODUCTION

In pancreatic *β*-cells, ATP-dependent potassium channels (K_{ATP}) have been reported to regulate insulin secretion by coupling the cell metabolism to the membrane excitability [1]. A key stimulus for insulin secretion is an increased blood glucose level that increases cytosolic ATP ($[ATP]_c$). However, this change is only a 30% to 40% above the basal ATP level [2]. The increase in $[ATP]_c$ in *β*-cells is believed to initiate the stimulus-secretion coupling cascade: promotes closure of K_{ATP} channels, membrane depolarization of the *β*-cell syncytia and opening of the voltage-activated calcium channels (VACC), together resulting in the elevation of cytosolic calcium and triggering the release of insulin-containing granules [3].

The K_{ATP} channel is formed by inward rectifier potassium channel 6.2 (Kir6.2) and sulphonylurea receptor (SUR1) subunits in a 4:4 stoichiometry [4,5]. The four Kir6.2 subunits comprise an inwardly rectifying K^+ channel while SUR1 subunits are regulatory proteins belonging to ABC transporter family [6]. SUR1 subunits conglomerate with Kir6.2 subunits in the endoplasmic reticulum and were suggested to chaperon the fully assembled K_{ATP} channels to the plasma membrane [7,8]. SUR1 subunit also confers K_{ATP} channels' response to pharmacological agents such as sulfonylureas and diazoxide [9], primarily defining the sensitivity of the K_{ATP} channels to ATP [10].

Human genetic studies have shown that mutations on both proteins forming the K_{ATP} channel impair functional insulin release and result in inappropriate serum insulin levels at a given plasma glucose level [11]. Gain of function mutations lead to hypoexcitability and suppress insulin release in neonatal diabetes mellitus (NDM) [12-14]. Conversely, loss of function of K_{ATP} channels leads to a persistent hyperexcitabilty of the *β*-cell and results in persistent hyperinsulinaemic hypoglycaemia of in-

fancy (PHHI) [15-17]. These patients commonly exhibit symptomatic hypoglycaemia soon after birth, are largely unresponsive to K_{ATP} channel openers such as diazoxide and often require subtotal (95%) resection of the pancreas to alleviate hypoglycaemia [18].

K_{ATP} channel-deficient mice have been generated by genetic disruption of Kir6.2 subunit (Kir6.2$^{-/-}$) [19] which can serve as a PHHI animal model to investigate the role of K_{ATP} channels in the glucose-induced insulin-secretion *in vivo* [19-22].

In this study we originally assessed the electrical and secretory activities in the Kir6.2$^{-/-}$ mice using a novel fresh pancreatic slice preparation [23], a method that avoids enzymatic and mechanical stress to β-cells during sample preparation and preserves the functionality and architecture of islets in the slice. Despite the predicted and previously reported hyperexcitability of Kir6.2$^{-/-}$ β-cells, the mice lacking this protein show an age dependent change in VACC densities and plasma glucose levels. In addition we this is the first report describing a change in exocytotic efficacy of the β-cells in Kir6.2$^{-/-}$ ablated mice. The results of this study contribute to our understanding of the pathophysiology of PHHI in human patients, which may lead towards a consideration of a less radical therapy and management of the PHHI patients.

2. MATERIALS AND METHODS

2.1. Animals

All animals were kept under standard housing conditions with free access to water and food. Experiments were conducted according to the regulations of our institutional and state committees on animal experiments (ZTE, University of Goettingen, and Faculty of Medicine University of Maribor). Homozygous Kir6.2$^{-/-}$ mice were maintained in C57/B6 background and wildtype C57/B6 were used as control (Phillips-University Marburg, Germany). Animals were euthanized by CO_2 followed by cervical dislocation. Immediately afterwards, blood samples were taken from the tail vein for glucose measurements. Blood glucose concentration was measured using the glucose oxidase method (OneTouch Ultra, Lifescan, USA).

Pancreatic slices were prepared as previously described [23]. Briefly, 2% gelling agarose (Seaplaque GTG agarose, BMA, Walkersville, Md., USA) melted in warm (37˚C) extracellular solution (ECS) was injected into the pancreatic duct to stabilize the tissue. After injection, the pancreas was cooled immediately with an ice-cold ECS and cut into 140 µm thick slices using vibratome (VT 1000 S, Leica, Nussloch, Germany). After slicing, the tissue slices were kept in ice-cold ECS, carbogenated with 95% oxygen and 5% carbon dioxide. Prior to electrophysiological experiments, the slices were incubated for at least 30 minutes at 32˚C in carbogenated ECS.

2.2. Solutions

Standard extracellular solution (ECS) consisted of the following (in mM): 125 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.25 Na_2HPO_4, 2 Na-pyruvate, 0.25 ascorbic acid, 3 myo-inositol, 6 lactic acid, 1 $MgCl_2$ and 2 $CaCl_2$. If not otherwise indicated, the glucose concentration of the ECS was 3 mM. All extracellular solutions were bubbled with carbogen (95% O_2, 5% CO_2) for at least 30 minutes to keep the pH at 7.3.

Experiments were performed with two different pipette filling solutions. The first intracellular solution (ICS1) was used to measure cellular electrical activity in the current-clamp mode and contained (in mM): 132 K-gluconate, 28 KCl, 10 HEPES (pH 7.2 with KOH), 2 $MgCl_2$, 0.05 EGTA and 4 $ATPNa_2$. The intracellular solution (ICS2) to measure Ca^{2+} currents and membrane capacitance (C_m) contained (in mM): 127 Cs-methanesulfonate, 8 CsCl, 10 HEPES (pH 7.2 with CsOH), 2 $MgCl_2$, 0.05 EGTA, 20 tetraethylammonium (TEA) chloride and 4 $ATPNa_2$. The osmolarity of all used solutions was adjusted to 300 +/− 10 mOsm. All chemicals used were purchased from Sigma (USA) unless otherwise indicated.

2.3. Electrophysiology

Glass pipettes were pulled on a horizontal pipette puller (P97; Sutter Instruments, USA) from borosilicate glass capillaries (GC150F15; WPI, USA) to a resistance of 2 - 4 MΩ in a KCl-based solution. The perfusion chamber was mounted on an upright microscope (Eclipse E600FN, Nikon, Japan) equipped with a 60× water immersion objective (CFI Fluor, NA = 1). A CCD camera (Cohu, San Diego, CA, USA) was used for visualization. Fresh pancreas slices were held at the bottom of the recording chamber (400 µl) by a U-shaped platinum frame with a nylon-fiber grid and perfused continuously with carbogen-bubbled ECS (30˚C - 32˚C, 1.5 ml·min^{-1}). All experiments were performed in the standard whole-cell patch-clamp mode on a SWAM IIC dual-phase lock-in patch-clamp amplifier (Celica, Ljubljana, Slovenia). Cells from the second or third layer in the islets were used for electrophysiological recording to increase the probability of finding β-cells. β-cells were identified by their Na^+ currents inactivation pattern, the change in membrane potential or spiking electrical activity in the presence of elevated glucose [24,25] as well as the presence of gap junction conductance[26]. Recordings, stimulation, and basic analyses were performed using the WinWCP software (v3.52, John Dempster, University of Strathclyde, UK). The initial C_{slow} values were further used for the estimation of the surface area of the patched cells assuming a specific membrane capacitance (C_m) of 9 fF·µm^{-2}. All currents were analysed and presented after P/N leak current subtraction. To estimate changes in membrane capacitance (ΔC_m), the

piecewise-linear technique was used (sine wave frequency 1.6 kHz, amplitude 11 mV RMS). The deflection of the C_m trace in response to the 1 pF test pulse was used to calibrate the measured C_m values. C_m, access conductance (G_a), membrane current (I) and membrane potential (V) were recorded after filtering at 3 kHz and stored for off- line analysis. These data were transferred to a PC via an A/D converter (PCI-6035E, National Instruments, USA). Cells showing unstable holding currents or a leak conductance larger than 2 nS at basal conditions were excluded from the analysis. For data analysis and figure preparation we used Matlab, Matview (Matlab WinWCP extension, Wise Technologies, Ljubljana, Slovenia), Sigmaplot and Sigmastat (SPSS, Chicago, IL, USA). All values are presented as mean ± S.E.M and the data were compared by unpaired Student's *t*-test.

3. RESULTS

3.1. Age-Dependent Morphological and Physiological Changes of Pancreatic Islets in Kir6.2$^{-/-}$ Mice

Despite the genetic ablation of the K_{ATP} channel pore, the pancreatic islets in tissue slices obtained from 2 weeks old Kir6.2$^{-/-}$ mouse appeared normal with densely packed groups of cells (**Figure 1(a)**, bottom left). However, the islets of Kir6.2$^{-/-}$ mice aged 30 weeks and older showed hollow spaces with few or no cells and irregular outline (**Figure 1(a)**, bottom right). Moreover, this apparent change in the cyto-architecture also resulted in a lower yield of successful whole-cell recordings from Kir6.2$^{-/-}$ β-cells at older age. These finding are in agreement with previously published data that both the number and the intensity of insulin immunoreactivity of β-cells decrease as the Kir6.2$^{-/-}$ mice get older [19,22]. In addition, the probability to patch-clamp an α-cell in the central part of the islet was higher compared to young mice, also indicating that the population of α-cells within the islet has increased as also previously reported [22].

Besides the aforementioned morphological changes some physiological parameters we measured have also changed with the increasing age of the animals. When measuring blood glucose levels, we observed transient hypoglycaemia in young and progressive hyperglycaemia in old Kir6.2$^{-/-}$ mice. **Figure 1(b)** shows randomly measured blood glucose levels in control C57/B6 and Kir6.2$^{-/-}$ mice at different ages. Two to four weeks after birth, the Kir6.2$^{-/-}$ mice showed a mild, but significant hypoglycaemia with the mean glucose levels of 5.6 ± 0.2 mM (n = 29) compared to 8.5 ± 0.2 mM in age matched control mice (n = 27; $p < 0.001$). Similarly to previous reports, the mean glucose level in 5 - 28 week old Kir6.2$^{-/-}$ mice was comparable to C57/B6 controls with mean glucose level of 8.1 ± 0.3 mM and 8.3 ± 0.2 mM, respectively ($p = 0.6$). However, at ages of 30 weeks or older, Kir6.2$^{-/-}$ mice became significantly hyperglycaemic. The mean glucose level in Kir6.2$^{-/-}$ mice was 12.4 ± 0.5 mM (n = 26), while the mean glucose level in age-matched control C57/B6 mice was 8.5 ± 0.2 mM (n = 10; $p < 0.001$). This agedependent change in glucose homeostasis is in line with the previous evidence suggesting that it is caused by excessive β-cell death due to calcium toxicity or apoptosis [19].

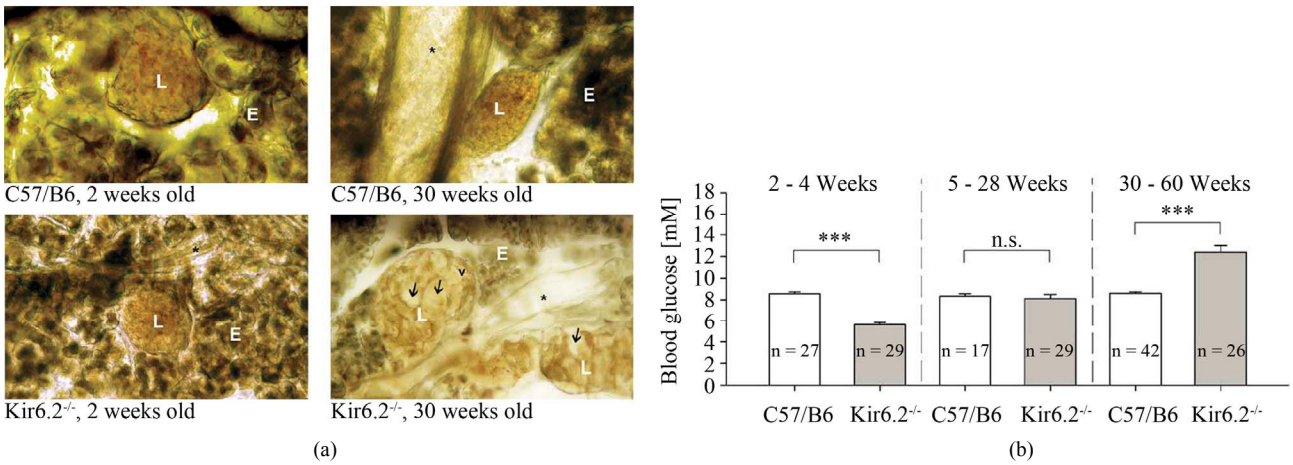

Figure 1. Transmitted light images of control C57/B6 and Kir6.2$^{-/-}$ mice islets in fresh pancreatic tissue slices and random blood glucose measurements: (a) The upper images show islets in fresh tissue slices from 2 and 30 weeks old C57/B6 mice. Note the intact islets of Langerhans (L) surrounded by the exocrine pancreas tissue (E). Longitudinally or transversely cut vessels and ducts were observed in the majority of slices (asterisks). Young Kir6.2$^{-/-}$ islets (lower left image) appeared intact and densely packed with cells, similar to control islets. Older islets (lower right) showed many hollow islets, containing connective tissue and few or no cells (arrows), with undefined borders and exposed fine vascularisation (v); (b) The random blood glucose measurement showed a transient mild hypoglycaemia in young Kir6.2$^{-/-}$ mice that progressed to relative hyperglycaemia in adult Kir6.2$^{-/-}$ mice (grey bars) compared to age-matched control mice (white bars). Numbers on bars indicate the number of mice for each experiment. n.s. $p > 0.05$; ***$p < 0.001$).

3.2. Electrophysiological Characterization of *β*-Cells in Slices

The *β*-cells have been identified using a standard protocol of 4 conditioning pre-pulses ranging from −150 to −40 mV. The characteristic inactivation properties of voltage-activated Na^+ channels in *β*-cells were found to be fully inactivated at −60 mV (**Figure 2(a)**). An additional criterion for *β*-cell identification was the presence of gap junction conductance, with *β*-cells unlike non-*β*-cells expressing gap junction conductance (**Figure 2(a)**). The third criterion was the glucose sensitivity of the electrical activity (**Figure 2(b)**, upper panel).

At low glucose concentrations (3 mM), wild-type *β*-cell syncytia are electrically silent with a resting membrane potential close to the equilibrium diffusion potential for K^+ [23]. At glucose levels above 6 mM, glucose-induced depolarization was sufficient to elicit repetitive bursting activity with action potentials superimposed on top of the bursts (**Figure 2(b)**, upper panel). In contrast, Kir6.2$^{-/-}$ *β*-cell syncytia displayed the electrical activity already at non-stimulatory glucose concentration (3 mM) (**Figure 2(b)**, bottom panel). Moreover, at this low glucose concentration, *β*-cell syncitia showed both bursting and spiking activity (**Figure 2(b)**, bottom panel inset). A major difference to wild-type syncytia is that action potentials were also present during the interburst periods. Addition of high glucose concentration to Kir6.2$^{-/-}$ slices did not further alter their electrical activity pattern. Among the 80 tested control C57/B6 *β*-cell syncytia, none of the cells showed spiking electrical activity in 3 mM glucose, while 82% of Kir6.2$^{-/-}$ *β*-cell syncytia showed both bursting as well as spiking activity at 3 mM glucose. The presence of slow bursting in Kir6.2$^{-/-}$ *β*-cell syncytia implies that there are still ion channels other than K_{ATP} channel on the plasma membrane that influence the membrane potential fluctuations.

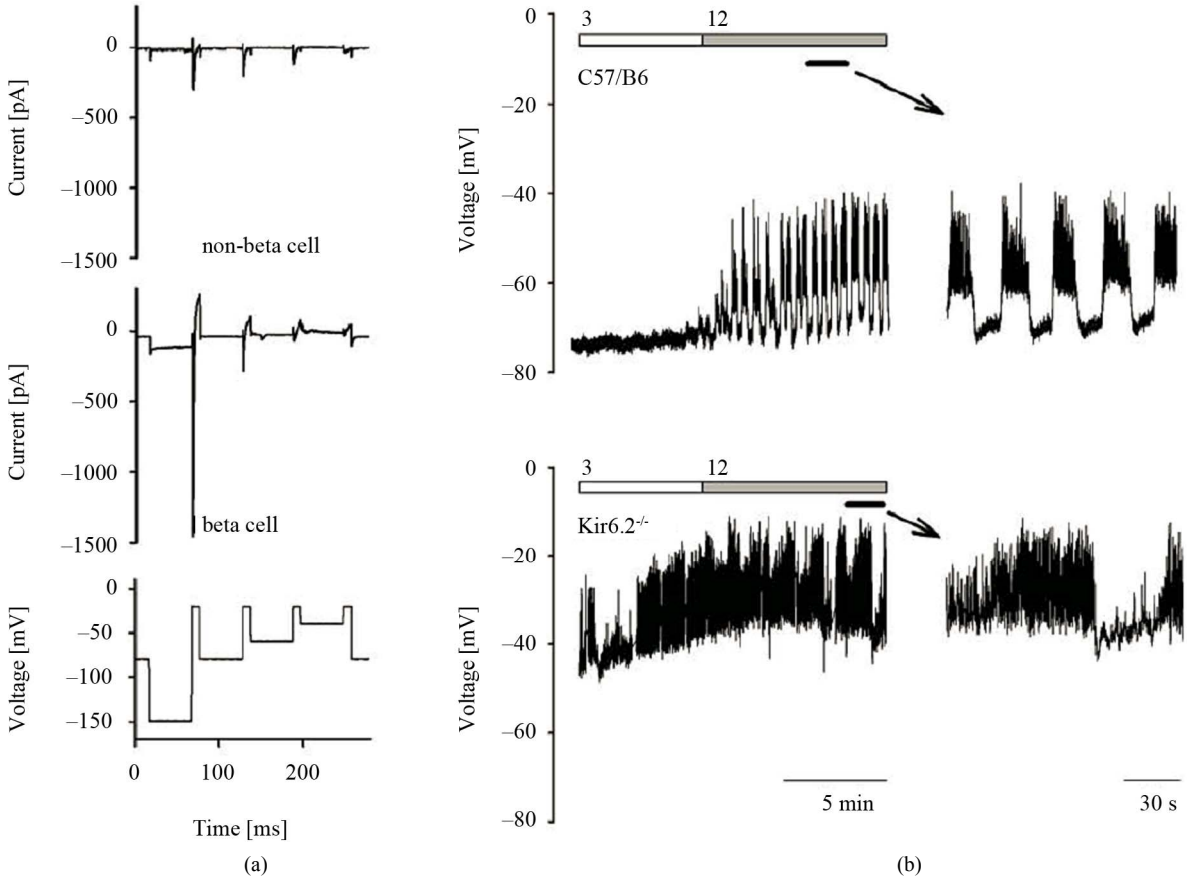

Figure 2. Cell identification and electrical activity in β-cells of control C57/B6 and Kir6.2$^{-/-}$ mice: (a) *β*-cells were identified based on the steady state inactivation characteristics of voltage gated Na^+ currents, gap junction conductance and (b) electrical activity in the presence of increased glucose. At 3 mM glucose, a control *β*-cell is electrically silent with a resting membrane potential of approximately –70 mV. At glucose levels above 6 mM electrical activity is initiated with its extent depending on the glucose concentration. In contrast, *β*-cells of Kir6.2$^{-/-}$ were electrically active already at 3 mM glucose. High glucose concentration did not independently alter the electrical activity in Kir6.2$^{-/-}$ mice. Note the oscillations of membrane potential in both cell types. Also note the individual electrical spikes between bursts of electrical activity in Kir6.2$^{-/-}$ *β*-cells (bottom panel). Numbers above bars indicate glucose concentration in mM.

Despite the overall increase in the size of the islets as the Kir6.2$^{-/-}$ mice become older, there was no significant difference in the *β*-cell size, measured as a resting membrane capacitance (C_m), a measure of cell surface area, in β-cells of Kir6.2$^{-/-}$ mice compared to age matched β-cells in control C57/B6 mice (**Figure 3(a)**). The mean resting C_m for Kir6.2$^{-/-}$ *β*-cells was 6.9 ± 1.7 pF (n = 45) and 6.7 ± 1.3 pF for control C57/B6 β-cells (n = 28; p = 0.6) (**Figure 3(a)**).

3.3. Voltage-Activated Ca^{2+} Currents in Kir6.2$^{-/-}$ and Control C57/B6 *β*-Cells

Insulin release from pancreatic *β*-cells is triggered by the entry of calcium ions through the VACCs. Modulation of the VACCs may therefore alter insulin exocytosis from *β*-cells [27]. We first compared the current size and current density of VACCs from *β*-cells of both genotypes. After subjection of the Kir6.2$^{-/-}$ and control C57/B6 *β*-cells to voltage ramps ranging from −80 to 60 mV with a duration of 300 ms (0.47 mV·ms^{-1}, **Figure 3(b)** 1) [28,29], two inward current components, showing peaks around −42 and −5 mV were identified (**Figure 3(b)** 2). These peaks corresponded to low (LVA) and high (HVA) voltage-activated Ca^{2+} currents. We found that Kir6.2$^{-/-}$ *β*-cells had larger voltage-activated Ca^{2+} current amplitudes, despite the persistent electrical activity. The pooled peak HVA Ca^{2+} currents were –175 ± 17 pA (n = 33) and –101 ± 6 pA (n = 25; p < 0.001), for Kir6.2$^{-/-}$ and control C57/B6 *β*-cells, respectively (**Figure 3(b)** 3, left). The pooled peak low-voltage activated (LVA) Ca^{2+} current amplitudes were –117 ± 12 pA (n = 33) and –64 ± 5 pA (n = 25; p < 0.001) for Kir6.2$^{-/-}$ and control C57/B6 *β*-cells, respectively (**Figure 3(b)** 4, left). Since the cell size of *β*-cells of the two phenotypes were comparable, we concluded that the VACC current densities were significantly larger in Kir6.2$^{-/-}$ *β*-cells compared to control C57/B6 *β*-cells (**Figure 3(b)** 3, right and **Figure 3(b)** 4, right). These larger VACC currents may contribute to the hypoglycaemic phenotype in young Kir6.2$^{-/-}$ mice.

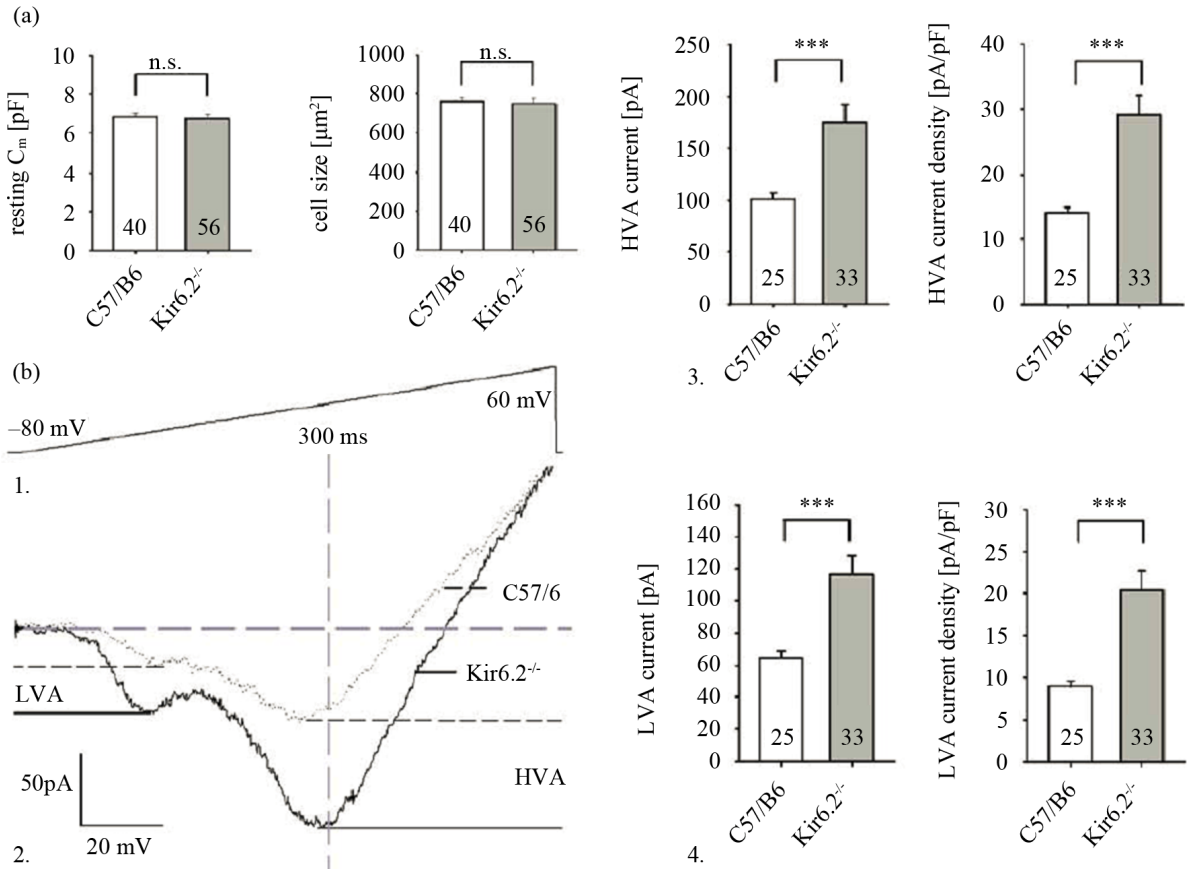

Figure 3. *β*-cell size and Ca^{2+} currents through VACC in control and Kir6.2$^{-/-}$ mice. (a) There is no significant difference in *β*-cells' total surface area or initial C_m of whole-cell patch-clamped β-cells of Kir6.2$^{-/-}$ (gray bars) compared to control C57/B6 mice (white bars). (b) 1. After subjection of *β*-cells to voltage ramps from −80 to 60 mV with duration of 300 ms (0.47mV/ms) 2. VACCs are readily activated in Kir6.2$^{-/-}$ (black line) and control *β*-cells (dotted line). 3. Peak HVA current and density and 4. peak LVA current and density in both phenotypes. Numbers on bars indicate the number of cells in each experiment. p < 0.001.

3.4. Stimulus-Secretion Coupling in β-Cells of Kir6.2$^{-/-}$ and Control C57/B6 Mice

In the β-cells of Kir6.2$^{-/-}$ mice we observed both hypo- and hyperglycaemia, depending on the age of the animals. To check how modified function of the secretory machinery in the β-cells from Kir6.2$^{-/-}$ animals may modify the glycaemia status in these animals, we performed a detailed examination of the stimulus-secretion coupling in both Kir6.2$^{-/-}$ and control C57/B6 β-cells. We used depolarization protocols to assess the size of the pool of the vesicles that are ready to exocytose.

In the whole-cell voltage-clamp mode, we applied a train of 50 depolarizing pulses of 40 ms duration from −80 to 10 mV at a frequency of 10 Hz (**Figure 4(b)** 1, top trace). **Figure 4(a)** shows histograms of the total C_m amplitude increase after such depolarizing train in control C57/B6 (**Figure 4(a)**, right) and Kir6.2$^{-/-}$ β-cells (**Figure 4(a)**, left). The distribution of C_m amplitudes from C57/B6 β-cells was unimodal peaking at about 100 fF. In contrast, the distribution of C_m amplitudes from Kir6.2$^{-/-}$ β-cells was bimodal, indicating two major groups of cells. The first peak (98 fF) resembled to the peak of the control group, while the second peak (251 fF) represented a subpopulation of β-cells with much larger capacitance increase (**Figure 4(a)**, right). Retrograde analysis of other physiological parameters measured in this study showed that the β-cells that peaked at higher C_m values were obtained from the hypoglycaemic mice typically younger than 4 weeks (**Figure 4(b)** 2).On the other hand, in β-cells from old hyperglycaemic Kir6.2$^{-/-}$ mice the C_m change has been significantly lower compared to the C_m change in β-cells from hypoglycaemic younger Kir6.2$^{-/-}$ mice (**Figure 4(b)** 4). Since we have noticed the heterogeneity of the depolarization train-induced C_m changes in Kir6.2$^{-/-}$ β-cells, we clustered the data into two groups: young (2 - 4 weeks old) and adult (5 - 60 weeks old) mice and statistically compared both phenotypes. The C_m changes were 110 ± 20 fF (n = 13) and 251 ± 29 fF (n = 16) for young control C57/B6 and Kir6.2$^{-/-}$, respectively (**Figure 4(b)** 4). These results indicate that the β-cells in young Kir6.2$^{-/-}$ mice may have a more efficient stimulus-secretion coupling than age-matched Kir6.2$^{+/+}$ mice. This finding could partially explain the hypoglycaemic phenotype in young Kir6.2$^{-/-}$ mice. These differences were not found in adult mice irrespective of the genetic background **Figure 4(b) 4**).

3.6. The Efficacy of Ca^{2+} to Trigger Secretion

Kir6.2$^{-/-}$ β-cells displayed a constitutive excitability at non-stimulatory glucose levels (**Figure 2(b)**, bottom panel). Calcium entry via VACCs has been suggested to be the major source of this ion to trigger exocytosis in β-cells [1]. High basal activity in Kir6.2$^{-/-}$ β-cells may result in an elevated basal calcium levels and thus increase the efficacy with which Ca^{2+} ions regulate the exocytotic machinery to produce a higher C_m change.

We quantified the calcium-dependent exocytotic efficacy in β-cells from both genotypes, since larger VACCs in β-cells from Kir6.2$^{-/-}$ mice may mask the impaired exo cytotic machinery, as was the case in β-cells from Goto Kakizaki (GK) rats shown previously[28]. We found that, despite of larger VACC in Kir6.2$^{-/-}$ β-cells, there was no difference in overall Ca^{2+} efficacy during the first train of depolarization compared to that of control C57/B6 β-cells. The mean efficacy ($\Sigma\Delta C_m/\Sigma\Delta Q$) of the first trains were 3.4 ± 0.3 fF pC^{-1} (n = 36) for Kir6.2$^{-/-}$ β-cells and 2.8 ± 0.3 fF pC^{-1} (n = 23) for control C57/B6 β-cells (**Figure 5(b)** 3).

In addition, we also applied a second train of depolarization pulses 4 min after the first train to assess any kind of an activity-dependent augmentation in β-cells from Kir6.2$^{-/-}$ due to residual Ca^{2+} from the first train [28]. In control C57/B6 β-cells, capacitance increases induced by the first and the second train were comparable in amplitude (115 ± 13 fF (n = 21) and 96 ± 14 fF (n = 21), respectively (p = 0.33) (**Figure 5(a)** 1 and 3), while in Kir6.2$^{-/-}$ β-cells, the second train of depolarization induced a much larger C_m increase compared with the first train (124 ± 17 fF (n = 30) and 232 ± 26 fF (n = 30), respectively; $p < 0.001$, (**Figure 5(a)** 2 and 3). Not surprisingly, for the second train of depolarization, the efficacy was higher in Kir6.2$^{-/-}$ β-cells compared to control C57/B6 β-cells (3.3 ± 0.5 fF pC^{-1} (n = 21) for control C57/B6 and 6.9 ± 0.7 fF pC^{-1} (n = 32) for Kir6.2$^{-/-}$ (**Figure 5(b)** 1 and 3).

4. DISCUSSION

A Kir6.2$^{-/-}$ mice have been originally produced as an animal model for congenital hyperinsulinaemia. The main novelty of this study is that we assessed the physiology of Kir6.2$^{-/-}$ β-cells in fresh pancreatic slice preparation in which cell-to-cell contacts, intercellular communication and tissue architecture are well preserved. In this study, we conclude that despite the predicted depolarized membrane potential in Kir6.2$^{-/-}$ β-cells, both the functional expression of VACCs as well as modified exocytotic efficacy contribute to the observed age-dependent reduction of the β-cell activity. These results may help us to better understand the pathophysiology of PHHI in human patients. More importantly, despite the fact that the expression of the K_{ATP} channels in β-cells seems to be the key element connecting glucose metabolism to insulin release, the loss of function mutations of the K_{ATP} channels or complete absence of the K_{ATP} channels may have an impact on time-dependency in the development of the phenotype. This time-dependent component should be considered in the design of the less robust therapeutic strategies for PHHI patients and other related human diseases.

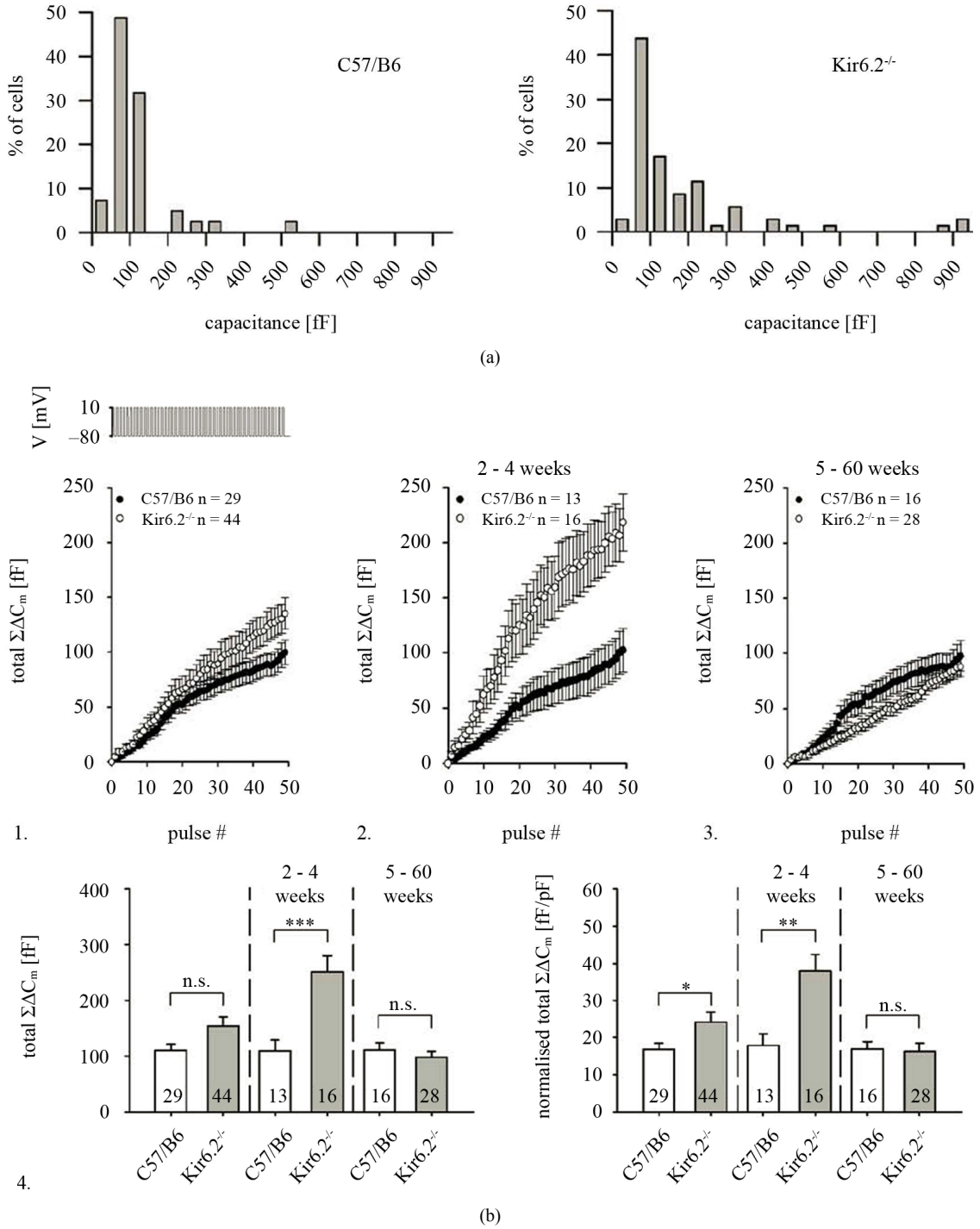

Figure 4. Stimulus-secretion coupling in *β*-cells of control and Kir6.2$^{-/-}$ mice. (a) Histogram of the total capacitance change in *β*-cells of control and Kir6.2$^{-/-}$ mice after a depolarization train; (b) 1-3 Depolarization train-induced changes in C_m in *β*-cells of control and Kir6.2$^{-/-}$ mice, obtained by subjecting *β*-cells to a train of 50 depolarisation pulses of 40 ms duration from −80 to 10 mV at a frequency of 10 Hz; 1. An average $\Sigma\Delta C_m$ during 50 depolarizations-train of control (closed circles) and Kir6.2$^{-/-}$ (open circles); 2. However, after separation into different age-groups, there was a significant difference between control (open circles) and Kir6.2$^{-/-}$ *β*-cells (closed circles) in a group of 2 - 4 weeks old mice 3. The group of 5 - 60 weeks old mice showed no differences; 4. The comparison of total $\Sigma\Delta C_m$ at the end of depolarization train (left) and total $\Sigma\Delta C_m$ normalised to cell size (right) between control *β*-cells (open bars) and Kir6.2$^{-/-}$ *β*-cells (closed bars) in different age groups as indicated. Numbers on bars indicate the number of mice for each experiment. $p > 0.05$; n.s. *$p < 0.05$; **$P < 0.002$; ***$p < 0.001$.

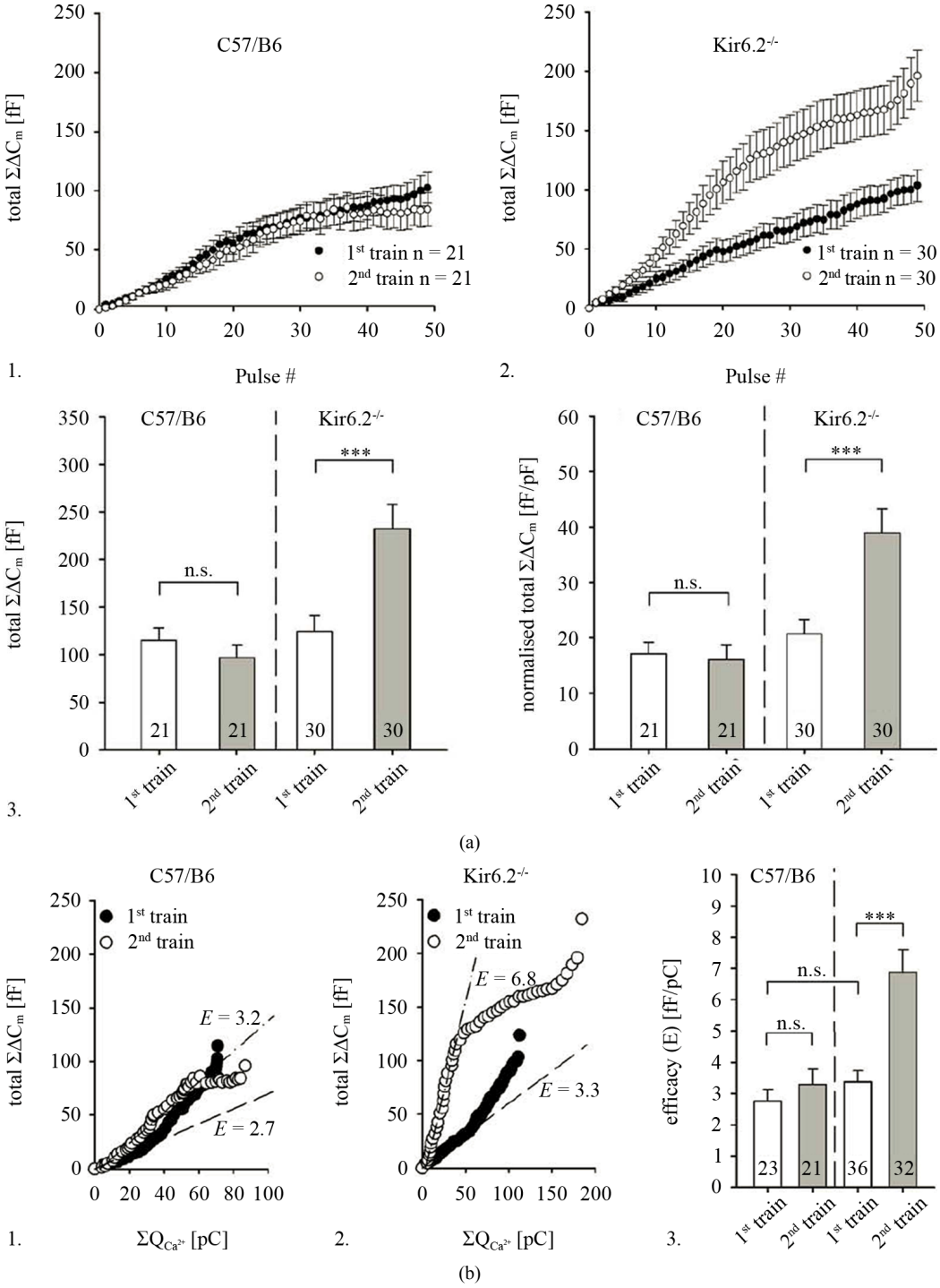

Figure 5. Secretion in β-cells during sequential depolarization train and calcium efficacy (E). (a) The average $\Sigma\Delta C_m$ in response to two successive depolarization trains in 1. control and 2. Kir6.2$^{-/-}$ β-cells, respectively (4 min interval between the trains). 3. comparison of the total $\Sigma\Delta C_m$ reached after the end of the train (left) and total $\Sigma\Delta C_m$ normalized to cell size (right) between control (open bars) and Kir6.2$^{-/-}$ (close bars) β-cells. Note the significant difference in total $\Sigma\Delta C_m$ between the successive trains in Kir6.2$^{-/-}$ β-cells; (b) Representative $\Sigma\Delta C_m$ as a function of $\Sigma\Delta QCa^{2+}$ of 1. control and 2. Kir6.2$^{-/-}$ β-cells, respectively. Straight lines represent linear fits through the first 10 data points (efficacy) of the 1st (dashed) and 2nd train (dot-dashed). 3. the average efficacy (E) of 1st and 2nd trains of control (left) and Kir6.2$^{-/-}$ β-cells (right). Note the significant difference between both trains in Kir6.2$^{-/-}$ β-cells. Numbers on bars indicate the number of cells in each experiment. n.s. $p > 0.05$; ***$p < 0.001$.

The gross appearance and the density of the β-cell packing in Kir6.2$^{-/-}$ mice younger than 4 weeks did not differ from the islets from control C57/B6 mice (**Figure 1(a)**). This result implies that K_{ATP} channels are not critical in the early postnatal morphogenesis, however on the long-term they seem to be indispensable for the survival of insulin-secreting β-cells. This is in agreement with previous studies that showed that hyperexcitability of the Kir6.2$^{-/-}$ β-cells may result in cell death or apoptosis due to calcium toxicity in aged mice [19].

Random blood glucose measurements in Kir6.2$^{-/-}$ mice showed a transient hypoglycaemia in young Kir6.2$^{-/-}$ mice (2 - 4 weeks old), normoglycaemia in young adult mice (5 - 28 weeks old), and a mild hyperglycaemia in aged mice (30 - 60 weeks old). Similar age dependencies have been shown in previous studies [19,20,22]. These changes are also likely to be caused by a reduction of β-cell mass in aged Kir6.2$^{-/-}$ mice. As reported previously, also our colony of Kir6.2$^{-/-}$ mice were obese as they became older [30].

At present, little is known about the role of the K_{ATP} channels in β-cell exocytosis. This report is the first attempt to assess complete lack of the K_{ATP} channels in this physiological process using pancreatic slice preparation. Capacitance measurements have been used in several experiments as secretory assay by indirectly measuring exo- and endocytosis involved in hormone secretion[23,28,31,32]. In Kir6.2$^{-/-}$ mice we found two subpopulations of β-cells; however it turned out that the smaller subpopulation which has shifted towards excessive C_m change in response to a train of depolarization pulses belonged exclusively to Kir6.2$^{-/-}$ mice younger than 4 weeks which were hypoglycaemic (**Figure 4(b)** 2). On the other hand, there were no significant differences in C_m changes between the β-cells from the adult mice (**Figure 4(b)** 3).

The excessive C_m response may be due to several reasons. First, in all insulin secreting cells from different rodent species tested so far, the Ca^{2+}-influx is mainly mediated by high voltage activated (HVA) L-type Ca^{2+} channels, either Cav1.2 or Cav1.3 channels [33]. In this study, we noticed a significantly larger HVA and LVA peak currents in Kir6.2$^{-/-}$ compared to control C57/B6 β-cells. Constitutive electrical activity, even in the presence of non-stimulatory glucose concentration and larger HVA current amplitude measured in Kir6.2$^{-/-}$ β-cells may be the cause of an elevated basal $[Ca^{2+}]_i$ measured in previous experiment [21]. Second, as in the fresh pancreatic slice preparation of GK rat β-cells [28], we found that C_m increase elicited by the second train of depolarizations had a significantly higher efficacy in Kir6.2$^{-/-}$ β-cells compared to control C57/B6, where this activity dependent augmentation did not take place (**Figure 5(b)**).

The question remains, why are the adult Kir6.2$^{-/-}$ mice normoglycaemic and in the last part of life even hyperglycaemic? The changed morphological features and previously published evidence suggest that this is mainly due to a progressively decreasing population of β-cells with increasing age in Kir6.2$^{-/-}$. In addition, many β-cells from aged Kir6.2$^{-/-}$ mice did not respond to a train of depolarizations indicating that there is an additional lesion in the secretory machinery.

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REFERENCES

[1] Ashcroft, F.M. and Rorsman, P. (1990) ATP-sensitive K^+ channels: A link between B-cell metabolism and insulin secretion. *Biochemical Society transactions*, **18**, 109-111.

[2] Maechler, P., Kennedy, E.D., Sebo, E., Valeva, A., Pozzan, T. and Wollheim, C.B. (1999) Secretagogues modulate the calcium concentration in the endoplasmic reticulum of insulin-secreting cells. Studies in aequorin-expressing intact and permeabilized ins-1 cells. *The Journal of Biological Chemistry*, **274**, 12583-12592 doi:10.1074/jbc.274.18.12583

[3] Ammala, C., Eliasson, L., Bokvist, K., Larsson, O., Ashcroft, F.M. and Rorsman, P. (1993) Exocytosis elicited by action potentials and voltage-clamp calcium currents in individual mouse pancreatic B-cells. *The Journal of Physiology*, **472**, 665-688.

[4] Inagaki, N., Gonoi, T., Clement, J.P., *et al*. (1995) Reconstitution of IKATP: An inward rectifier subunit plus the sulfonylurea receptor. *Science*, **270**, 1166-1170. doi:10.1126/science.270.5239.1166

[5] Shyng, S. and Nichols, C.G. (1997) Octameric stoichiometry of the KATP channel complex. *The Journal of General Physiology*, **110**, 655-664. doi:10.1085/jgp.110.6.655

[6] Aguilar-Bryan, L., Nichols, C.G., Wechsler, S.W., *et al*. (1995) Cloning of the beta cell high-affinity sulfonylurea receptor: A regulator of insulin secretion. *Science*, **268**, 423-426. doi:10.1126/science.7716547

[7] Schwappach, B., Zerangue, N., Jan, Y.N. and Jan, L.Y. (2000) Molecular basis for K(ATP) assembly: Transmembrane interactions mediate association of a K^+ channel with an ABC transporter. *Neuron*, **26**, 155-167. doi:10.1016/S0896-6273(00)81146-0

[8] Zerangue, N., Schwappach, B., Jan, Y.N. and Jan, L.Y. (1999) A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K(ATP) channels. *Neuron*, **22**, 537-548. doi:10.1016/S0896-6273(00)80708-4

[9] Inagaki, N., Gonoi, T., Clement, J.P., *et al*. (1996) A

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