UNIVERSITE CATHOLIQUE DE LOUVAIN
Secteur des Sciences de la Santé
Unité d'Endocrinologie et Métabolisme

ROLE OF THE BETA-CELL CYTOSKELETON IN METABOLIC AND NEUROHORMONAL AMPLIFICATION OF INSULIN SECRETION

Nizar I. MOURAD

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Table of contents

Abbreviations

ABP	actin binding protein
AC	adenylate cyclase
ACC	acetyl-CoA carboxylase
ACh	acetylcholine
AICAR	5-aminoimidazole-4-carboxamide riboside
AMPK	AMP-activated protein kinase
Ca_v	voltage-dependent calcium channel
$[Ca^{2+}]_c$	free cytosolic Ca^{2+}concentration
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP-GEFII	cAMP-regulated protein guanine nucleotide exchange factor
CPT1	carnitine palmitoyltransferase 1
DAG	diacylglycerol
Dz	diazoxide
Epac2	exchange protein directly activated by cAMP 2
ER	endoplasmic reticulum
ERM	ezrin, radixin, moesin
F-actin	filamentous actin
FFAR1	free fatty acid receptor 1
Fsk	forskolin
G-actin	globular actin
GIP	glucose-dependent insulinotropic peptide
GK	glucokinase
GLP-1	glucagon-like peptide-1
Glut1, Glut2	glucose transporter 1, 2
GPR	G-protein-coupled receptor
IBMX	3-isobutyl-1-methylxanthine
INS-1	insulin-secreting rat cell line
IP_3	inositol-1,4,5-trisphosphate
Jasp	jasplakinolide
K-ATP channel	ATP-dependent potassium channel

Kir6.2	K^+ inward rectifier 6.2
K_v	voltage-dependent K^+ channels
LCFA-CoA	long chain fatty acyl CoA
MAP	microtubule-associated protein
MARK	microtubule affinity-regulating kinase
MIN6	insulin-secreting mouse cell line
MLCK	myosin light-chain kinase
mTOR	mammalian target of rapamycin
NCX	sodium/calcium exchanger
PDE	phosphodiesterase
PEPCK	phosphoenolpyruvate carboxykinase
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKA	proteine kinase A
PKC	proteine kinase C
PLC	phospholipase C
PMA	4 beta-phorbol myristate acetate
PMCA	plasma membrane Ca^{2+}-ATPase
RRP	readily releasable pool
SERCA	sarco-endoplasmic reticulum calcium ATPase
SNAP25	synaptosomal-associated protein 25
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SOC	store-operated current
SOCE	store-operated calcium entry
SUR1	sulphonylurea receptor 1
TIRFM	total internal reflection fluorescence microscopy
Tolb	tolbutamide
t-SNARE	target SNARE
VAMP-2	vesicule-associated membrane protein 2
VDCC	voltage-dependent calcium channel
v-SNARE	vesicule SNARE

I. INTRODUCTION

Glucose homeostasis, i.e. maintaining blood glucose concentrations within a relatively narrow range, is an important, life-protecting process in humans. Thus, exceedingly low blood glucose rapidly endangers brain function and can ultimately lead to coma and death, whereas too high blood glucose can acutely cause severe life-threatening hydro-saline disorders and chronically damage blood vessels, nerves and kidneys, the major sites of complications in uncontrolled diabetes.

The regulation of blood glucose is achieved by a very efficient hormonal system which exerts opposite effects on glucose-producing and glucose-storage organs. Whereas several hormones (glucagon, noradrenaline, cortisol …) can prevent inappropriate declines in blood glucose by stimulating glycogenolysis and gluconeogenesis in the liver, insulin secretion by beta-cells of the islets of Langerhans is the only means by which the organism can decrease glycemia. It is therefore not surprising that insulin secretion is subject to a tight control ensured by glucose itself and by an array of metabolic, neural and hormonal factors as will be described in chapter II.

Disturbances of glucose homeostasis most commonly occur in type 2 diabetes, the prevalence of which is increasing at an epidemic rate throughout the world, linked to the rise in prevalence of obesity. Epidemiological studies agree on estimations that the number of cases worldwide has approximately doubled between 2001 and 2010, to reach 220 million, and predict a further increase to more than 400 million in 2030 (Shaw et al. 2010, Nolan et al. 2011). The hyperglycemia of type 2 diabetes results from insufficient insulin secretion in a context of diminished insulin action in target tissues (Cerasi 1995, Kahn et al. 2009). Knowing how the normal beta-cell works is thus essential for elucidation of its defects in diabetes.

A large number of studies over the last five decades have led to our current understanding of stimulus-secretion coupling in beta-cells. The picture that emerges is that of a dual regulation of insulin secretion by both triggering and amplifying signals produced by glucose (Henquin 2009). It is undisputed that a rise in the

concentration of free cytosolic calcium ($[Ca^{2+}]_c$) in beta-cells is the critical signal for triggering insulin secretion upon glucose stimulation. The consensus model ascribes this rise to the acceleration of beta-cell metabolism, a subsequent increase of the ATP/ADP ratio, closure of ATP-sensitive K^+ (K-ATP) channels, membrane depolarization with activation of voltage-gated Ca^{2+} channels and influx of Ca^{2+} into the cell. The action of Ca^{2+} on exocytosis of insulin granules is then augmented by still incompletely identified amplifying signals acting on unknown targets. To provide the background of my studies, a description of this series of events and of their effectors is presented in chapter III, in which I also develop our current understanding of the amplifying pathway and of the most pertinent mechanisms proposed to be involved in this pathway.

Chapter IV describes and discusses the biphasic pattern of glucose-induced insulin secretion. The mechanisms underlying this peculiar kinetics remain an intellectual challenge for cell biologists. Their elucidation would also be of interest for clinicial diabetologists who remain intrigued by the selective disappearance of the first phase during the earliest stages of development of type 2 diabetes. The chapter will also set the stage for my studies on the contribution of the amplifying pathway to the two phases.

Chapter V reviews previous studies regarding the characteristics of beta-cell microfilaments and microtubules, and the role of the cytoskeleton in insulin secretion. It will serve as an introduction to my studies.

Finally, chapter VI summarizes and discusses the results of my research which focused on the involvement of the amplifying pathway in the first and second phases of insulin secretion, and on the possibility that cytoskeleton-dependent granule recruitment is the step of stimulus-secretion coupling affected by metabolic amplification.

II. STIMULUS RECOGNITION BY PANCREATIC BETA-CELLS

An array of nutrients, hormones and neuroendocrine signals control the secretion of insulin, the body's only glucose-lowering hormone, in order to ensure optimal regulation of blood glucose levels. This chapter briefly outlines how beta-cells recognize these extracellular signals and respond by increasing or decreasing the rate of insulin secretion.

1-Glucose

Unlike most secretory cells that identify their stimuli through selective receptors, pancreatic beta-cells do not express glucose receptors. Glucose must be metabolized for the beta-cell to secrete insulin, and the rate of secretion is governed by the rate of metabolism. Numerous studies have shown that non-metabolized analogs of glucose do not induce insulin secretion and that the response to glucose is inhibited whenever oxidative phosphorylation is uncoupled (Sener and Malaisse 1984, Matschinsky 1996).

In rodents, glucose enters beta-cells by facilitated diffusion via the high capacity, low-affinity glucose transporter Glut2 (Thorens et al 1988, Newgard et al. 1995). The high Km (15-20 mM) of this transporter permits efficient glucose uptake in proportion to blood glucose levels, so that glucose concentrations rapidly equilibrate across the beta-cell membrane (Matschinsky 2009). This is also the case in human beta-cells although Glut1, which has a lower Km, is the predominantly expressed isoform of glucose transporters (De Vos et al. 1995, Ferrer et al. 1996, Richardson et al. 2007).

Upon its entry into beta-cells, glucose is phosphorylated by a glucokinase (GK, hexokinase IV) that acts as a glucose sensor due to its high Km and lack of inhibition by its product, glucose-6-phosphate (Matschinsky 1996). The key role of GK is highlighted in human pathology. Activating mutations of the enzyme cause certain forms of congenital hyperinsulinism with neonatal hypoglycemia, whereas

inactivating mutations impair insulin secretion and lead to diabetes (Matschinsky 2002). Following its phosphorylation, glucose almost exclusively follows the glycolytic pathway leading to the formation of ATP, NADH and pyruvate.

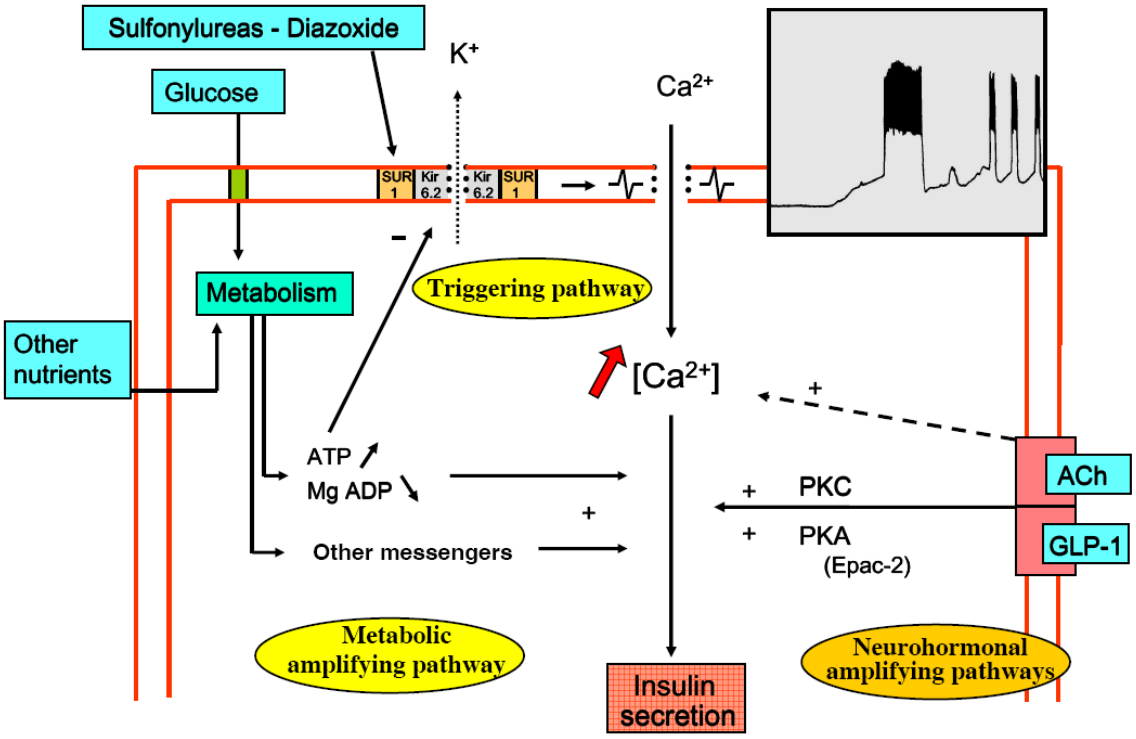

Fig. 1: Schematic representation of stimulus-secretion coupling in beta-cells. Triggering and metabolic amplifying pathways mediate the stimulation of insulin secretion by glucose. Neurohormonal amplifying pathways mediate the effects of neurotransmitters and hormones. The K_{ATP} channel, made up of SUR1 and Kir6.2 subunits, is the transducer of metabolic changes into membrane potential changes. The inset illustrates the depolarisation and the electrical activity induced by glucose in one mouse beta cell within an intact islet; the recording was made with an intracellular electrode. The dotted line indicates a decreased flow. +, stimulation; −, inhibition; SUR1, sulfonylurea receptor 1; Kir, inwardly rectifying K channel; ACh, acetylcholine; Epac-2, exchange protein directly activated by cAMP 2; GLP-1, glucagon-like peptide 1; PKA, protein kinase A; PKC, protein kinase C.
Taken from Henquin 2009.

Owing to the low expression of lactate dehydrogenase (MacDonald et al. 2005a), which converts pyruvate to lactate and reoxidizes NADH into NAD^+ in most cells, and of plasma membrane monocarboxylate transporters (Zhao et al. 2001), which permit pyruvate exit, more than 90% of pyruvate is channeled to the mitochondria. In addition, two particularly active shuttles permit electron transfer

between cytosolic NADH and mitochondria: the glycerol phosphate and the malate-aspartate shuttle systems (MacDonald 1982).

In mitochondria, more NADH is produced from pyruvate entering the TCA cycle via pyruvate dehydrogenase. Electrons are then transferred from these reduced coenzymes to the electron transport chain leading to pumping of H^+ ions out of the mitochondrial matrix, hyperpolarization of the inner mitochondrial membrane (Duchen et al. 1993) and ATP generation by ATP-synthase. Moreover, mitochondrial inner membrane hyperpolarization stimulates potential-dependent Ca^{2+} entry into mitochondria (Litsky and Pfeiffer 1997) thereby increasing Ca^{2+}-dependent dehydrogenase activity and augmenting NADH and ATP production from the TCA cycle (Hansford et al. 1991). These features of glucose metabolism in the beta-cell eventually lead to an increase in the cytosolic ATP concentration. Previous studies from the laboratory have measured a change from ~3.0 to ~4.7 mM ATP in whole mouse islets incubated at 2 and 20 mM glucose (Detimary et al. 1996a). At present, ATP produced by glucose oxidation is considered to be the main second messenger coupling glucose entry to insulin granule exocytosis, but it is probably not the only one.

Another peculiarity of glucose metabolism in the beta-cell is the high activity of pyruvate carboxylase which converts about 50% of pyruvate entering the mitochondria to oxaloacetate (MacDonald et al. 1993). Pharmacological inhibition of pyruvate carboxylase reduces glucose-induced insulin secretion (Wiederkehr and Wollheim 2008) supporting the importance of the anaplerotic branch of beta-cell pyruvate metabolism. This anaplerotic activity adds new carbons to the TCA cycle and allows some of the cycle intermediates to exit the mitochondria without compromising cycle function. These TCA cycle intermediates such as glutamate, citrate and malate are exported to the cytosol where they may serve as additional mitochondrial signals by producing second messengers involved in the control of insulin secretion such as long chain fatty acyl CoA. Several correlative studies point towards a role of malonyl CoA (Corkey et al. 1989) and NADPH (Ashcroft and Christie 1979, Hedeskov et al. 1987, Ivarsson et al. 2005b) but there still is no widely

accepted model for the action of these messengers. A recent study also suggests that the relative importance of distinct metabolic pathways may differ between human and rodent islets (MacDonald et al. 2011).

Apart from its central role in controlling insulin secretion, glucose also promotes insulin synthesis by stimulating proinsulin gene expression (Permutt and Kipnis 1972, Andrali et al. 2008) and by enhancing proinsulin mRNA stability (Fred and Welsh 2009). However, the stimulation of preproinsulin mRNA translation is of even greater importance and accounts for the increase in insulin biosynthesis observed during short-term (0-3 hours) glucose stimulation (Goodge and Hutton 2000). These effects of glucose also require metabolism of the sugar in beta-cells, but are not yet fully understood (Melloul et al. 2002). In this context, it is important to emphasize that short-term control of insulin secretion does not depend on insulin synthesis, since islet insulin stores largely exceed secretory rates.

2-Amino acids

Unlike glucose, no amino acid *alone* increases insulin secretion when it is used at a physiological concentration (75 µM arginine in plasma vs. 2-10 mM *in vitro*). Amino acids become effective at supraphysiological concentrations or when they are used in combination or in the presence of glucose. Leucine is metabolized in beta-cell mitochondria to produce acetyl-CoA (Malaisse et al. 1986) that enters the TCA cycle thus producing ATP and increasing the cytosolic ATP/ADP ratio. Glutamine alone does induce insulin secretion. However, in the presence of leucine, which activates glutamate dehydrogenase, glutamate (formed from glutamine) is metabolized into α-ketoglutarate which fuels the TCA cycle (Newsholme et al. 2010). These effects are thus similar to those of glucose.

Other amino acids increase insulin secretion without affecting the ATP/ADP ratio. Their action involves changes in beta-cell membrane potential due either to their co- transport with Na^+, as it is the case with alanine (Prentki and Renold 1983), or their positive electrical charge as for arginine and other cationic amino acids

(Henquin and Meissner 1981). Some disputed evidence, however, suggests that alanine metabolism can increase insulin secretion by generating glutamate, aspartate and lactate and that arginine-derived nitric oxide (NO) can have a negative effect on insulin release (Newsholme et al. 2010). Leucine, but not the other amino acids, also stimulates insulin synthesis, probably via activation of the mTOR pathway (Yang et al. 2010).

3-Fatty acids

Fatty acids do not acutely increase insulin secretion in the presence of low glucose. The most probable reason is that, although well oxidized in beta-cells, they do not increase the ATP/ADP ratio, do not depolarize the membrane and hardly increase $[Ca^{2+}]_c$ (Warnotte et al. 1994). In the presence of stimulatory glucose concentrations, fatty acids augment insulin secretion by several mechanisms.

As stated earlier, citrate, a TCA cycle intermediate generated by the anaplerotic branch of pyruvate metabolism in the mitochondria, is exported to the cytosol and converted to malonyl-CoA which can be used in the synthesis of lipids (MacDonald et al. 2005b). Moreover, through its inhibition of palmitoyltransferase 1 (CPT1), malonyl-CoA decreases fatty acid transport into mitochondria as well as their oxidation and leads to an increase of cytosolic LCFA-CoAs. The involvement of these molecules in the regulation of insulin secretion is still debated (Mulder et al. 2001) and will not be discussed further. The provision of exogenous free fatty acids increases the intracellular concentrations of LCFA-CoA and other lipid signals which might contribute to the increase in insulin secretion (Nolan et al. 2006). LCFA-CoAs can be converted to diacylglycerol (DAG) which stimulates signaling via PKC (MacDonald et al. 2005b).

In addition to their metabolic effects, fatty acids also induce insulin secretion by binding to specific G-protein-coupled transmembrane receptors (GPRs), notably FFAR1 also known as GPR40 (Kebede et al. 2009). The activation of these receptors induces phospholipase C (PLC)-dependent hydrolysis of phosphatidylinositol 4,5

bisphosphate into diacylglycerol (DAG) and inositol trisphosphate (IP3) followed by activation of protein kinase C (PKC) (Kebede et al. 2009), mobilization of intracellular Ca^{2+} (Shapiro et al. 2005) and therefore an increase in secretion.While this fatty acid receptor activation explains the small effect on $[Ca^{2+}]_c$ in low glucose, it is not sufficient to induce insulin secretion; glucose-dependent activation of plasma membrane Ca^{2+} channels and metabolism are also required.

4-Hormones and neurotransmitters

Numerous hormones and neuropeptides can increase or decrease insulin secretion (Ahrén 2009). These effects are sometimes very species-dependent and their physiological relevance is not always established. These issues are beyond the scope of my thesis, and I will only briefly allude to a few major members of this long list.

4.1-Hormonal signals

Glucagon-like-peptide-1 (GLP-1) is secreted by L-cells from the mucosa of the ileum and colon, and glucose-dependent insulinotropic polypeptide (GIP) is secreted by K-cells from the duodenojejunal mucosa. These two hormones bind to their respective G protein-coupled receptors (GPCRs) (Ahrén 2009) and are responsible for the incretin effect that occurs during meals and is experimentally demonstrated by the larger stimulation of insulin secretion when a given glucose load is administered orally rather than being intravenously injected (Baggio and Drucker 2007, Vislsboll and Holst 2004). The effect of these two hormones is mainly mediated by cAMP acting through protein kinase A and Epac2 (Fig. 1) as will be described in greater detail in chapter VI.

Glucagon secreted by islet alpha-cells also increases glucose-induced insulin secretion by activating cAMP-dependent pathways via its own receptors, distinct from those mediating the effect of GLP-1 (Gromada et al. 1997).

Adrenaline secreted by the adrenal medulla during stress, hypoglycemia or physical activity binds to α_2-receptors in beta-cells and inhibits insulin secretion by decreasing cAMP, hyperpolarizing the plasma membrane and inhibiting distal steps of exocytosis (Lang et al. 1995).

4.2-Neural signals

Pancreatic islets are innervated by parasympathetic, sympathetic and sensory nerves. Thus, insulin secretion is stimulated by the parasympathetic and inhibited by the sympathetic system, respectively (Ahrén 2000). Acetylcholine (ACh) is the main neurotransmitter involved in parasympathetic control of beta-cells. The cephalic phase, i.e. insulin secretion in response to sensory stimuli prior to any increase in blood glucose levels, depends on parasympathetic stimulation that is then sustained during the whole meal. The net effect of ACh is a synergic increase of glucose-induced insulin secretion mainly via activation of phosphoinositide hydrolysis by PLC which results in the formation of IP_3 and DAG that induce mobilization of intracellular Ca^{2+} stores and PKC activation, respectively (Fig. 1). ACh also causes membrane depolarization and thereby increases Ca^{2+} influx (Gilon and Henquin, 2001). Further discussion of these effects can be found in chapter VI.

Activation of the sympathetic system results in inhibition of insulin secretion due to noradrenaline release and its binding to α_2-adrenoreceptors on the beta-cell (Nakaki et al. 1980). This inhibition occurs via the same mechanisms as those involved in adrenaline effects.

III. GLUCOSE-INDUCED INSULIN SECRETION

1-Triggering pathway

Glucose-induced insulin secretion requires glucose metabolism by beta-cells and depends on a rise in $[Ca^{2+}]_c$ due to the influx of extracellular Ca^{2+}. This corresponds to the triggering pathway of insulin secretion that I will detail below after a brief description of the basis of electrical excitability and Ca^{2+} homeostasis in beta-cells.

1.1-Beta-cell membrane electrical activity

Pancreatic beta-cells are electrically excitable (Dean and Mathews 1968) owing to the presence of voltage-dependent ion channels in their plasma membrane. In response to glucose, they depolarize because the acceleration of metabolism decreases membrane K^+ conductance (Henquin 1978). The main actors of this phenomenon are ATP-sensitive K^+ channels (K-ATP) which were found to be the missing link between glucose metabolism and Ca^{2+}-induced insulin secretion. These channels, discovered in guinea pig cardiac muscle (Noma 1983), were then identified (Cook et al 1984; Ashcroft 1984) and cloned in beta-cells (Aguilar-Bryan and Bryan 1995). K-ATP channels have a tetrameric organization with four subunits of an inwardly rectifying K^+ channel (Kir6.2) which forms the pore to which ATP binds to close the channel, surrounded by four subunits of a high-affinity sulfonylurea receptor (SUR1) to which ADP binds to open the channel.

At substimulatory glucose concentrations, the beta-cell plasma membrane potential is set around -70 mV by the hyperpolarizing K^+ outward current flowing through K-ATP channels. Closure of these channels in response to glucose metabolism leads to a gradual decrease of K^+ conductance. Since the IC_{50} value for K-ATP channel inhibition by ATP is in the range of 5-25 µM and the cytosolic ATP concentration amounts to 3-5 mM, it has been argued that K-ATP channels would be

permanently closed if they sensed $[ATP]_c$. However, the models of beta-cell electrical activity predicting 99% closure of K-ATP channels at physiological nucleotide concentrations rely on measurements in excised membrane patches (Proks and Ashcroft 2009, Rorsman et al. 2011).

Whole-cell measurements have shown that only 5-10% of K-ATP channels are open at substimulatory glucose concentrations (< 6-7 mM in mice) and that the ATP sensitivity of the channels is markedly shifted towards higher ATP concentrations in intact cells as compared with membrane patches (Proks and Ashcroft 2009, Rorsman et al. 2011). One possible explanation is that ATP-consuming pumps lower [ATP] in the vicinity of the channels (Drews et al. 2010), thus allowing glucose-induced changes in beta-cell ATP/ADP ratio (from ~3 to ~10 between 1 and 10 mM glucose) (Detimary et al. 1998a) to function as a signaling pathway controlling K-ATP channel activity. Both the decrease in ADP and the increase in ATP thus affect the activity of K-ATP channels resulting in closure of these channels and a decrease in plasma membrane permeability to K^+.

Closure of K-ATP channels alone is not enough to cause membrane depolarization. When enough K-ATP channels are closed, the electrical resistance of the membrane becomes high enough to permit depolarization by background inward currents (Ashcroft 2005, Henquin et al. 2009, Rorsman et al. 2011). The exact nature of this depolarizing current is still unknown and it is unlikely to involve one single ionic pathway (Henquin et al. 2009, Rorsman et al. 2011). The current could result from Na^+ and/or Ca^{2+} influx (Hiriart and Aguilar-Bryan 2008) through transient receptor potential channels (Henquin et al. 2009, Islam 2010) or from Cl^- efflux (Best 2005).

As shown in Fig. 2, the beta-cell membrane slowly depolarizes in response to glucose stimulation. When the membrane potential reaches -50 mV, further rapid depolarization occurs to a plateau onto which spike shaped action potentials appear (Henquin and Meissner 1984; Drews et al. 2010). These action potentials are provoked solely by Ca^{2+} currents in mice while Na^+ currents contribute to this electrical activity in rats and humans (Drews et al. 2010, Braun et al. 2008). After a

first long train of spikes, the membrane partly repolarizes and beta-cells display a characteristic pattern of electrical activity known as “slow waves” (Henquin and Meissner 1984b, Drews et al. 2010) consisting of bursts of action potentials and hyperpolarized interburst phases. Termination of the bursts of action potentials is caused by repolarizing currents generated by different types of K^+ channels (Henquin 1990, MacDonald and Wheeler 2003, Dufer et al. 2009a, Drews et al. 2010, Rorsman et al. 2011). This complex oscillatory electrical activity is orchestrated by several types of ion channels, the identity and contribution of which are still debated between electrophysiologists. Inactivation of voltage-gated Ca^{2+} channels, activation of Ca^{2+}-activated K^+ channels (SK) or large-conductance Ca^{2+}-activated K^+ channels (BK), depolarizing store-operated channels (Drews et al. 2010, Rorsman et al. 2011) and TRPM5 channels (Brixel et al. 2010, Colsoul et al. 2010) have all been proposed to contribute to the shaping of glucose-induced electrical activity in beta-cells.

I will simply emphasize that the oscillations of membrane potential are subject to modulation by the concentration of glucose: the depolarized phases with action potentials last longer and the interburst phases become shorter with increasing glucose concentrations, and continuous electrical activity appears when the concentration of glucose exceeds 20-25 mM (Fig. 2). The early proposal that this increase in electrical activity is mainly achieved by progressive closure of more and more K-ATP channels (Henquin 1988) has often been challenged by experiments performed in single cells, but has received direct support from observations that a significant proportion of the channels remain active between 5 and 25 mM glucose in beta-cells within intact islets (Rorsman et al. 2011).

1.2-Calcium homeostasis in beta-cells

Like most other cells, non-stimulated beta-cells maintain their resting $[Ca^{2+}]_c$ between 50 and 100 nM despite slight but continuous Ca^{2+} entry down its electro-chemical gradient. This is achieved by active transport of Ca^{2+} out of the cell or by Ca^{2+} sequestration in intracellular compartments notably the endoplasmic reticulum (ER). Three major mechanisms permit Ca^{2+} extrusion from the cytoplasm upon $[Ca^{2+}]_c$ elevation:

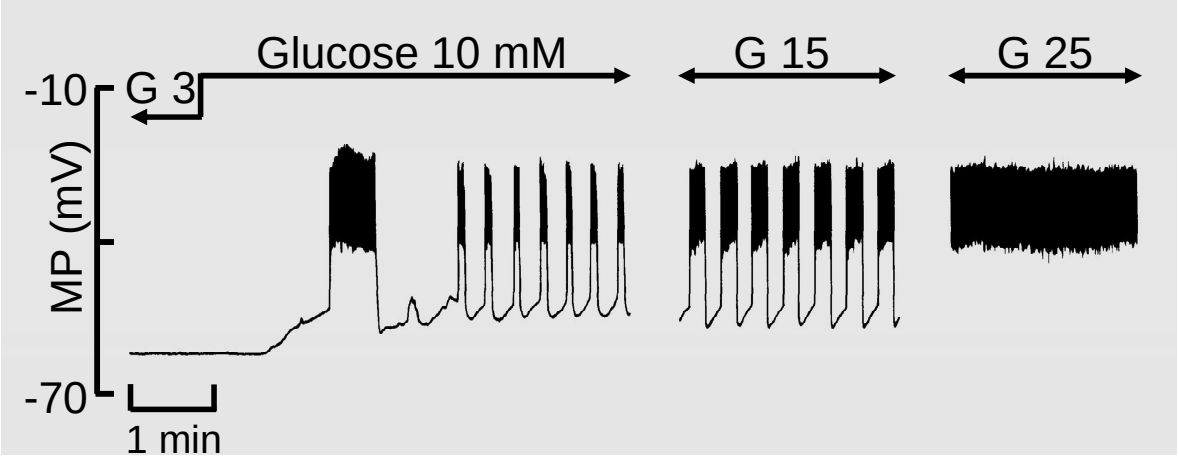

Fig. 2: Beta-cell membrane potential changes in response to increasing glucose concentrations. In the presence of 3 mM glucose, the resting membrane potential (MP) is stable around -65 mV. Following stimulation with 10 mM glucose, MP slowly rises to a threshold where rapid depolarization occurs. MP then returns to more negative values and displays oscillations on the plateau of which appear bursts of action potentials. These burst periods last longer when glucose is raised from 10 mM to 15 mM (G15). At 25 mM glucose (G25), MP displays continuous bursting activity (taken from Henquin 1990).

• Ca^{2+} is pumped out of the cell by a plasma membrane calmodulin-sensitive Ca^{2+}-ATPase (PMCA). Of the several isoforms of this protein, PMCA 1b and 2b are expressed in beta-cells and the other islet cells whereas PMCA 4b is specifically expressed in beta-cells (Varadi et al. 1996, Herchuelz et al. 2007).

• Ca^{2+} extrusion is also achieved by countertransport of one Ca^{2+} in exchange for four Na^{+} by a Na^{+}/Ca^{2+} exchanger (NCX) driven by the electrochemical gradient of Na^{+}. Splice variants NCX1.3 and NCX1.7 are the major isoforms expressed in beta-cells (Herchuelz et al. 2007).

• Ca^{2+} is also cleared from the cytosol by pumping into the ER by a sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA2b and SERCA3) (Prentki et al. 1984). Glucose can affect replenishment of ER Ca^{2+} stores in the beta-cell by increasing both ATP and $[Ca^{2+}]_c$ (Tengholm et al. 1999, Arredouani et al. 2002b, Varadi and Rutter 2002). Moreover, it has been shown that while SERCA2b is involved in basal $[Ca^{2+}]_c$ regulation, SERCA3 only becomes operative when $[Ca^{2+}]_c$ rises and is involved in glucose-induced $[Ca^{2+}]_c$ oscillations (Arredouani et al. 2002a).

1.3-Glucose-induced $[Ca^{2+}]_c$ changes

Upon stimulation by glucose, there initially occurs a slight decrease in $[Ca^{2+}]_c$ (Fig. 3A and B) attributed to Ca^{2+} sequestration in the ER (Tengholm et al 1999). This effect of glucose is indeed prevented by SERCA inhibition using thapsigargin (Chow et al. 1995). Acceleration of the pumping rate of SERCA2b is tentatively attributed to a greater availability of ATP (because of metabolism acceleration) (Arredouani et al. 2002a). Thus, no initial decrease precedes the rise in $[Ca^{2+}]_c$ produced by non-metabolized agents such as tolbutamide (Fig. 3C and D). The functional significance of this Ca^{2+} sequestration by the ER is unknown. A teleological explanation is that it prepares beta-cells to respond to hormones and neurotransmitters which will mobilize Ca^{2+} from the ER (Hiriart and Aguilar-Bryan 2008). Alternatively, it could play a role in shaping the oscillatory pattern of $[Ca^{2+}]_c$ changes observed during sustained glucose stimulation by modulating SOC currents (Arredouani et al. 2002b, Drews et al. 2010).

Following the initial decrease, $[Ca^{2+}]_c$ increases from a basal value of approximately 50 nM to a peak of about 250 nM within about one minute. This increase in $[Ca^{2+}]_c$ is essential for stimulating insulin secretion (Grodsky and Bennett 1966, Henquin 1978). Antagonism of voltage-dependent Ca^{2+} channels (VDCC) is sufficient to inhibit insulin secretion in humans and rodents (Drews et al. 2010). These channels, which permit entry of extracellular Ca^{2+} down its concentration gradient, are major actors in stimulus-secretion coupling. VDDCs or Ca_v channels comprise a pore-forming α1 subunit and several modulatory auxiliary subunits (Drews et al. 2010). In pancreatic beta-cells, several isoforms of Ca_v channels classified according to the structure of their α1 subunit are expressed and it is still a matter of debate which ones are of physiological relevance. There is, however, general agreement that L-type channels (Ca_v1.1-1.4) play a key role in beta-cell stimulus secretion coupling. These slowly inactivated channels are capable of achieving long-lasting changes in $[Ca^{2+}]_c$ required for insulin secretion (Drews et al. 2010). There is, however, evidence for the contribution of other types of VDCC to glucose-induced Ca^{2+} influx in human beta-cells (Rorsman et al. 2011). Intracellular

Ca^{2+} store release has been suggested to play a role in regulating insulin secretion. This release can be secondary to the influx of extracellular Ca^{2+}, as it is with Ca^{2+}-induced Ca^{2+} release from the ER (Graves and Hinkle 2003, Tengholm and Gylfe 2009) or induced by external signals which modulate insulin secretion rather than trigger it. In general, the current view holds that the major determinant of the glucose-induced increase $[Ca^{2+}]_c$ is extracellular Ca^{2+} influx through VDCCs.

Following the initial peak, $[Ca^{2+}]_c$ slowly decreases to a nadir, still well above basal levels, and then starts to oscillate. These oscillatory changes mirror the pattern of membrane potential with different types of $[Ca^{2+}]_c$ oscillations occurring in response to glucose (Henquin 2009). The period of these oscillations ranges from several seconds to several minutes (Bertram et al. 2007) thus allowing their subdivision into ultrafast, fast and slow oscillations. Sometimes, fast oscillations are superposed on slow ones creating a pattern of compound oscillations often observed in single beta-cells, beta-cell clusters and isolated islets in response to glucose stimulation (Beauvois et al. 2006, Fridlyand et al. 2010). One point on which there is general agreement is the dependency of these oscillations on Ca^{2+} influx since they are abrogated in the absence of extracellular Ca^{2+} or by blockade of L-type Ca^{2+} channels (Tengholm and Gylfe 2009).

Some authors proposed that the intrinsic oscillatory activity of the glycolytic enzyme phosphofructokinase 1 (PFK-1) (Tornheim 1997) leads to metabolic oscillations that in turn cause $[Ca^{2+}]_c$ oscillations. Another point of view is that these metabolic oscillations are secondary to $[Ca^{2+}]_c$ oscillations: the glucose-induced increase in $[Ca^{2+}]_c$ reduces the mitochondrial membrane potential ($\Delta\psi$) which then diminishes ATP production, leading to reopening of some K-ATP channels, partial membrane repolarization and a lowering $[Ca^{2+}]_c$ due to VDCC closure (Drews et al. 2010). Alternatively, each increase in $[Ca^{2+}]_c$ could increase ATP consumption, lead to a decrease in the ATP/ADP ratio and eventually permit reopening of a few K-ATP channels (Detimary et al. 1998b). Finally, these oscillations might be inherent to the nature of the ionic channels: $[Ca^{2+}]_c$ changes could thus create a feedback loop that

fine-tunes the activity of VDCCs and K_{Ca} and results in periodic activation/deactivation of these channels (Rorsman et al. 2011).

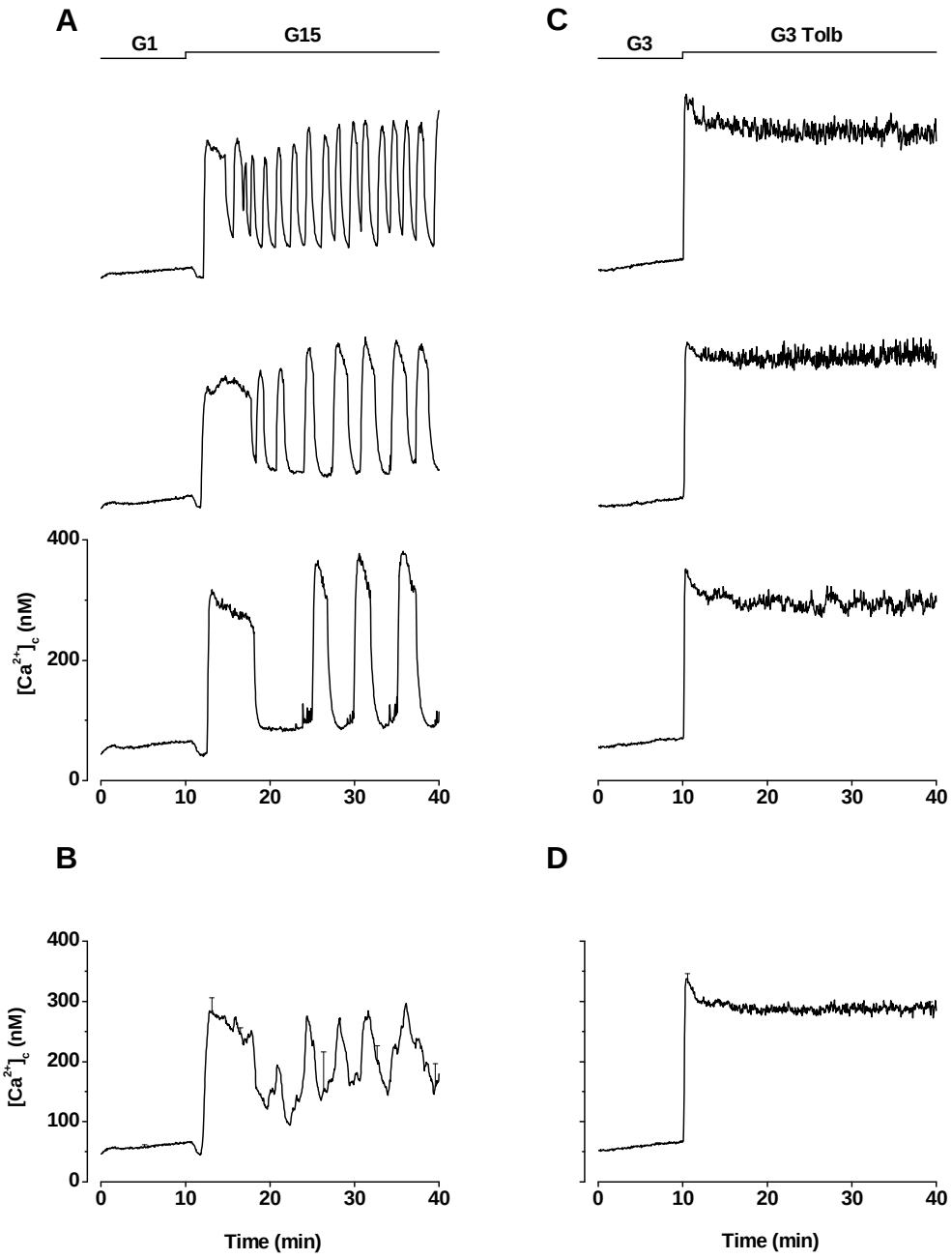

Fig. 3: Glucose and tolbutamide-induced $[Ca^{2+}]_c$ changes in isolated mouse islets.
A and C: Individual responses of 3 islets from the same mouse showing non synchronized $[Ca^{2+}]_c$ oscillations in response to glucose (A) and a stable and sustained plateau in response to tolbutamide (C). B and D: Mean responses of 6 islets to glucose (B) and to tolbutamide (D). Note the dampened changes obtained after averaging the responses to glucose (B) due to lack of synchrony between oscillations in individual islets (personal results).

1.4-How does Ca^{2+} trigger exocytosis?

As in many other secretory cells, insulin granule exocytosis is mediated by the SNARE (soluble NSF attachment protein receptor; NSF, N-ethylmaleimide-sensitive factor) protein complex. SNAREs belong to a large family of proteins mediating intracellular fusion between various compartments of the cell. They are subdivided into vesicular SNAREs (v-SNAREs): synaptobrevin/VAMP-2 attached to the secretory granule membrane and target SNAREs (t-SNAREs): syntaxin and SNAP-25 attached to the plasma membrane (Becherer and Rettig 2006). SNARE protein pairing creates a core complex that bridges the vesicular and plasma membranes bringing them in close proximity and inducing their fusion. The completion of this assembly reaction requires a rise in $[Ca^{2+}]_c$ and thus necessitates a Ca^{2+} sensor for signal transduction.

The v-SNARE protein synaptotagmin is a major putative Ca^{2+} sensor (Lang 1999, Sorensen 2004, Eliasson et al. 2008). Most synaptotagmins are anchored to the secretory vesicle through their N-terminal domain and bind Ca^{2+} with micromolar affinity through their cytoplasmic C2 domains which also mediate the oligomerization of synaptotagmin as well as its attachment to the assembled SNARE complex (Becherer and Rettig 2006). At least three isoforms, synaptotagmin 5, 7 and 9, have been detected in primary beta-cells. It is likely that these different isoforms with different affinities for Ca^{2+} are responsible for the difference in Ca^{2+} sensitivity found between distinct populations of insulin granules. Synaptotagmin 9 is coupled to fast neurotransmitter release in neurons and might constitute the low affinity Ca^{2+} sensor in beta-cells (Iezzi et al 2005), but this view has recently been challenged (Gustavsson et al 2010). Synaptotagmin 5 or 7 may represent the high affinity Ca^{2+} sensor (Eliasson et al. 2008).

Calmodulin is another protein known for its involvement in membrane fusion events such as Ca^{2+}-dependent exocytosis (Quetglas et al. 2002). The Ca^{2+}-bound form of calmodulin has a high affinity for several signaling molecules such as protein kinases (MLCK, CaMKII) and phosphatases (calcineurin) and can thus affect different players of the exocytotic machinery.

Besides mediating the molecular events of exocytosis, the SNARE complex is functionally coupled to the excitatory machinery through its association with VDCCs, creating microdomains surrounding the granule attachment sites and ensuring optimal exposure of the exocytotic components to high $[Ca^{2+}]_c$ (Eliasson et al. 2008).

In order for the granule cargo to be delivered to the extracellular medium, fusion between the secretory granule and the plasma membrane must occur. This fusion can take place in two ways. The simplest and most known form of exocytosis is "full fusion exocytosis" where the granule membrane is completely integrated in the plasma membrane with complete release of the whole content. This is usually followed by recycling of the vesicle membrane via endocytosis (Sokac and Bement 2006). This type of full fusion is thought to account for more than 90% of the secretory response in beta-cells (Ma et al. 2004, Kasai et al. 2010). Alternatively, a narrow fusion pore can be formed through which small molecules of low molecular weight (Zn^{2+}, ATP, Ca^{2+}, glutamate, serotonin, dopamine) but not insulin can pass. In this very quick type of fusion, termed "kiss-and-run" exocytosis, it is possible that low molecular weight substances are released to exert a regulatory function within the islet (Rorsman and Renstrom 2003).

1.5-Importance of K-ATP channels for the triggering pathway

The preceding paragraphs have summarized the major steps by which glucose leads to Ca^{2+}-induced insulin secretion (triggering pathway). This consensus model thus places K-ATP channels at the centre of the stimulus-secretion coupling mechanism.

The model is strongly supported by pharmacological evidence that agents known to directly affect K-ATP channel activity produce dramatic changes in insulin secretion. Hypoglycemic sulfonylureas, such as the prototypic tolbutamide and glibenclamide, prevent the activating action of ADP via their binding to SUR1 and thereby provoke closure of K-ATP channels, depolarization, a rise in $[Ca^{2+}]_c$ and an increase in insulin secretion even in low glucose (Henquin 2000, Drews et al. 2010).

More recent sulfonylurea compounds and non-sulfonylurea derivatives, such as glinides, have similar effects and are commonly used in the treatment of type 2 diabetic patients. Conversely, diazoxide counteracts glucose-stimulated insulin secretion by directly opening K-ATP channels without decreasing beta-cell metabolism. This hyperglycemic drug, sometimes used for the treatment of hyperinsulinism and certain insulinomas, binds to the SUR1 subunit and stabilizes the open state of the channel (Henquin 2000, Gribble and Reimann 2003).

The importance of K-ATP channels is also highlighted by certain pathologies of insulin secretion in which mutations in either of the two K-ATP channel subunits lead to uncoupling of glucose metabolism from beta-cell electrical activity. In congenital hyperinsulinemic hypoglycemia of infancy, mutations in *Kir6.2* and, more often, of *Sur1* result in incorrect channel assembly or lack of channel trafficking to the plasma membrane. This loss of K-ATP channel activity leads to constitutive membrane depolarization and persistent insulin release, regardless to the blood glucose level (Aguilar-Bryan and Bryan 1999, Dunne et al. 2004). Conversely, gain-of-function mutations in Kir6.2 are associated with neonatal diabetes. In this case, the mutation results in a reduction of the ATP sensitivity of Kir6.2 leading to insulin secretion defects and hyperglycemia (Ashcroft 2007, Clark and Proks, 2010).

1.6-Shortcomings of the K-ATP channel model

It is undeniable that the K-ATP-mediated triggering pathway is a major component of glucose-induced secretion, but several lines of evidence suggest that other pathways and mechanisms might also contribute to the rise in $[Ca^{2+}]_c$.

Ca^{2+}-induced Ca^{2+} release from the ER was thus suggested to contribute to the rise in $[Ca^{2+}]_c$ (Tengholm and Gylfe 2009). Moreover, the filling state of the ER Ca^{2+} stores may control Ca^{2+} entry across the plasma membrane through store-operated Ca^{2+} entry (SOCE) (Islam 2010).

Other suggestions are based on experiments using islets lacking K-ATP channels due to a knockout of one of the channel subunits. In islets from *Sur1* knockout mice for example, beta-cells are depolarized and their $[Ca^{2+}]_c$ is elevated in

the presence of low non-stimulatory concentrations of glucose (Nenquin et al. 2004, Dufer et al. 2007). Surprisingly, however, these islets respond to high glucose stimulation by changing the frequency of the oscillations of membrane potential and $[Ca^{2+}]_c$, which results in a rise in the triggering signal. Under certain conditions, glucose can even produce a biphasic increase in $[Ca^{2+}]_c$. (Szollosi et al. 2007). Similar observations have been made in *Kir6.2*KO islets (Ravier et al. 2009). These unexpected findings in islets lacking functional K-ATP channels raise the possibility that channels other than K-ATP can transduce the glucose signal into electrical and ionic changes in beta-cells. The nature of these channels and whether they are involved in normal beta-cells or represent an adaptation in K-ATP-lacking cells remain to be determined.

2-Amplifying pathway

The first suggestions that glucose can increase insulin secretion by mechanisms independent of its action on K-ATP channels are more than 20 years-old (Henquin 1988, Panten et al. 1988). Direct supporting evidence was obtained a few years later (Gembal et al. 1992, Sato et al. 1992).

This non-electrogenic effect of glucose first referred to as the "K-ATP channel-independent pathway" and now more rightfully named the "amplifying pathway" (Henquin 2000) increases insulin secretion beyond what could be accounted for by electrical coupling and Ca^{2+} signaling described in the previous paragraphs. Since its discovery in rodent islets, the amplifying pathway has been found in pig and human islets and in some insulin-secreting cell lines (Henquin et al. 2009) and has thus gained wide acceptance among beta-cell specialists.

This additional pathway operates independently of the activation status of K-ATP channels. Another trademark is that it does not involve further changes in $[Ca^{2+}]_c$ than those produced by the triggering pathway. The amplifying pathway requires elevated $[Ca^{2+}]_c$ (induced by any stimulus) but the marked increase in insulin secretion that it brings about is not accompanied by an increase in $[Ca^{2+}]_c$. This view

was based on measurements of global $[Ca^{2+}]_c$ changes throughout the cytosol (Gembal et al 1993), but a recent study also excluded changes in $[Ca^{2+}]$ in a microdomain just underneath the plasma membrane (Ravier et al. 2010). Glucose is thus able to increase the efficacy of Ca^{2+} on exocytosis, hence the use of the term "amplifying".

Like the triggering pathway, the amplifying pathway requires glucose metabolism (Gembal et al 1993, Henquin 2009) hence the adjective "*metabolic*" used to describe it and distinguish it from "*neurohormonal*" amplification that is set in operation after binding of a ligand to a membrane receptor. Interference with glucose metabolism inhibits insulin secretion by suppressing both triggering and amplifying signals. Moreover, nutrients other than glucose (amino acids) whose metabolism increases the ATP/ADP ratio also exert an amplifying effect (Gembal et al. 1993, Sato and Henquin 1998). Metabolic amplification is second only to the triggering pathway in hierarchy because it manifests itself only once $[Ca^{2+}]_c$ elevation has been triggered by glucose or another stimulus. Quantitatively, however, it is as important as the triggering pathway, often contributing to more than 50% of the total secretory response (Henquin et al. 2009). This contribution can be detected in different ways.

2.1-Amplification when K-ATP channels are held open by diazoxide

This experimental paradigm (Gembal et al. 1992, Sato et al. 1992) uses diazoxide to prevent glucose from closing K-ATP channels. Under these conditions, high concentrations of glucose do not induce secretion because the plasma membrane cannot be depolarized and $[Ca^{2+}]_c$ does not increase. However, the triggering Ca^{2+} signal can be generated by increasing the concentration of extracellular K^+, a maneuver that brings the membrane potential to more positive values thus permitting opening of voltage-dependent Ca^{2+} channels. Beta-cell depolarization by high K^+ causes a similar rise in $[Ca^{2+}]_c$ in the presence of low or high glucose (Fig. 4A) but insulin secretion is much larger in high glucose (Fig. 4B). This difference in the secretory response to a similar triggering signal (produced here by K^+) corresponds to the amplifying effect of glucose. Incidentally, diazoxide is necessary to prevent

glucose from affecting beta-cell electrical activity and $[Ca^{2+}]_c$ in these experiments performed in the presence of KCl (Gembal et al. 1992).

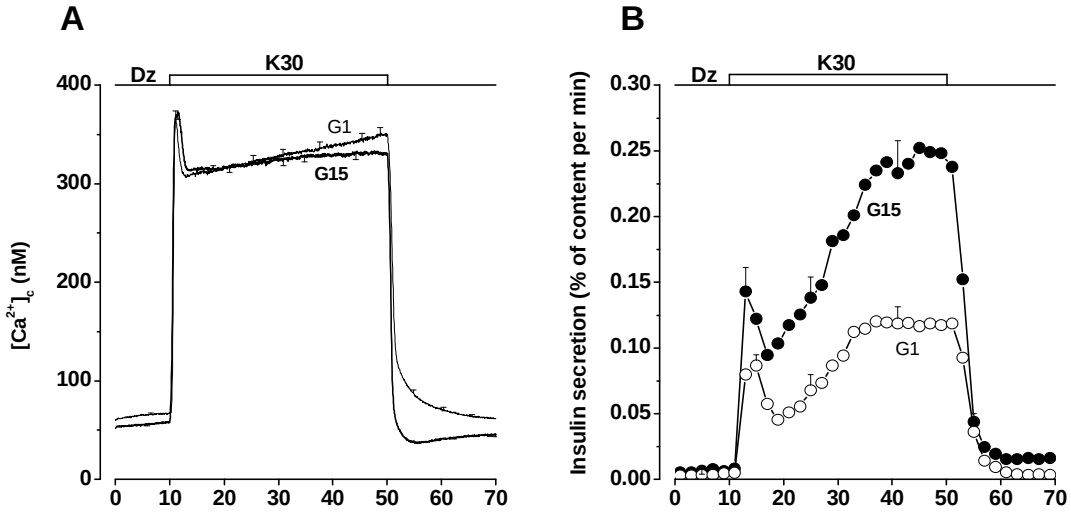

Fig. 4: Glucose amplifies KCl-induced insulin secretion.
$[Ca^{2+}]_c$ (A) and insulin (B) responses in mouse islets depolarized with 30 mM KCl in the presence of 100 µM diazoxide and either 1 mM glucose (G1; open circels) or 15 mM glucose (G15; filled circles). The results are means ± SEM of n=11 experiments in A and n=9 experiments in B (personal results).

2.2-Amplification when K-ATP channels are closed by tolbutamide

Closing all K-ATP channels with a maximally effective concentration of tolbutamide is another way to generate the triggering signal in the presence of substimulatory concentrations of glucose (Sato et al. 1999). As shown in figure 5A this is sufficient to induce insulin secretion. If the glucose concentration is then raised in the presence of tolbutamide, insulin secretion markedly increases (~ 4-fold) in the face of an only slight increase in $[Ca^{2+}]_c$ (Fig. 5B). This effect of glucose is thus largely attributable to the amplifying pathway. The transient decrease in $[Ca^{2+}]_c$ that initially occurs when the concentration of glucose is increased in the presence of tolbutamide (Fig. 5A) is due to Ca^{2+} uptake by the ER and transient membrane repolarization (with decrease in Ca^{2+} influx) because of a decrease in store-operated depolarizing currents (Dukes et al. 1997)**.** Interestingly, this transient decrease of $[Ca^{2+}]_c$ is not accompanied by a similar decrease in secretion as shown in figure 5B. This can be explained by the insufficient temporal resolution in most insulin secretion measurement experiments (a small, transient decrease of secretion can

sometimes be seen in individual experiments) but also by the rapid onset of metabolic amplification of secretion.

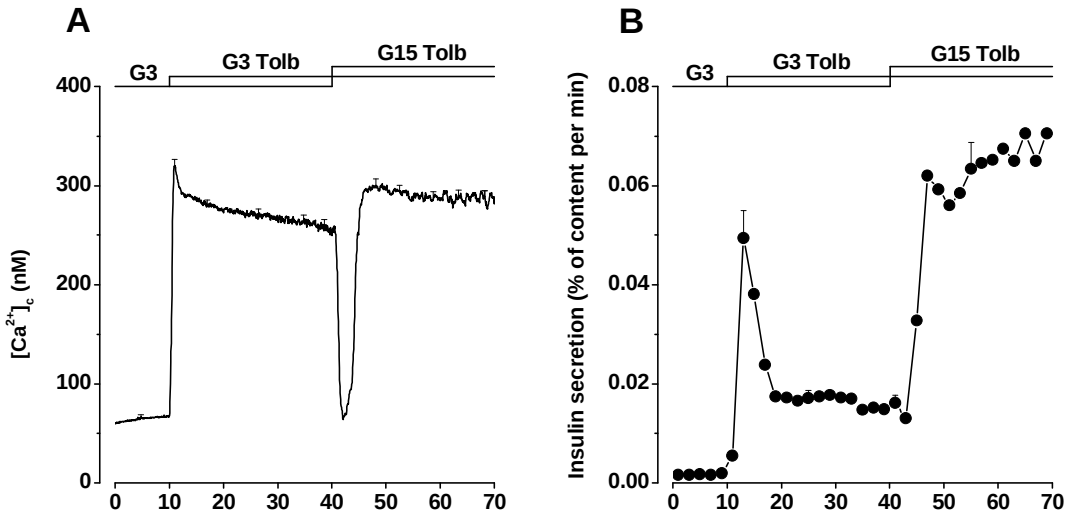

Fig. 5: Glucose amplifies tolbutamide-induced insulin secretion.
Stimulation with tolbutamide (Tolb; 500 µM) in the presence of substimulatory glucose (3 mM=G3) triggers a rapid and sustained increase of $[Ca^{2+}]_c$ (A) and a biphasic insulin response (B). Increasing glucose to 15 mM (G15) causes a transient decrease in $[Ca^{2+}]_c$ followed by a return to values slightly higher than in the presence of 3 mM glucose. The decrease in insulin secretion is hardly seen due to the insufficient temporal resolution of the perifusion system. Most importantly, insulin secretion is about 4 times higher in the presence of 15 mM glucose (B) while $[Ca^{2+}]_c$ is only minimally higher (A) (personal results).

2.3-Amplification in models lacking K-ATP channels

Islets lacking K-ATP channels because of a knockout of *Sur1* or *Kir6.2* show elevated Ca^{2+} and secrete insulin in the presence of non-stimulatory concentrations of glucose (Nenquin et al. 2004). Several laboratories, including ours, also reported that high glucose increases insulin secretion from these islets (Eliasson et al. 2003, Haspel et al. 2005, Szollosi et al. 2007, Ravier et al. 2009), but this was not observed by all groups (Miki et al. 1998, Seghers et al. 2000, Shiota et al. 2005). I have already mentioned that glucose can influence the triggering Ca^{2+} signal in these islets (see 1.6), but the increase in insulin secretion is essentially due to amplification. Intriguingly, no similar increase was observed in the pancreas of infants suffering from congenital hyperinsulinism because of inactivating mutations of K-ATP channels (Henquin et al. 2011).

2.4-Amplification at the single beta-cell level

Cell capacitance is proportional to the cell surface area. Capacitance measurements monitor the changes in cell surface area that occur when secretory granules fuse with the plasma membrane. Exocytosis of a secretory vesicle thus produces a certain increase in capacitance and such measurements can therefore be used to monitor secretory events at the single cell level with very high time resolution (Rorsman and Renstrom 2003). Using this technique, it has been shown that glucose increases the exocytotic response evoked by membrane depolarization 5-fold over that observed in the absence of the sugar (Eliasson et al. 1997).

Another group used two-photon excitation imaging to visualize exocytotic events in islets or clusters of beta-cells. The technique detects filling of vesicles undergoing exocytosis by a polar compound present in the extracellular milieu (Kasai et al. 2010). It was found that the number of exocytotic events induced by an increase in $[Ca^{2+}]_c$ (photolysis of caged Ca^{2+}) was more than doubled by pretreatment with 20 instead of 2.8 mM glucose (Hatakeyama et al. 2006). Glucose thus rapidly increased Ca^{2+}-induced insulin secretion. These two sets of results added further proof that glucose stimulates exocytosis by a late effect that is distal to plasma membrane depolarization.

2.5-Amplification under physiological conditions

To establish the physiological significance of the amplifying pathway, experiments were needed that did not involve clamping of beta-cell $[Ca^{2+}]_c$. This was achieved by comparing the increases in $[Ca^{2+}]_c$ and insulin secretion which occurred in mouse islets stimulated by various concentrations of glucose (Henquin et al. 2006b). A sigmoidal relationship was found between the glucose concentration and both $[Ca^{2+}]_c$ and insulin secretion changes, but the response curve for insulin was consistently shifted to the right compared with that of $[Ca^{2+}]_c$. This shift means that glucose produces signals other than the rise in $[Ca^{2+}]_c$ to induce insulin secretion.

Another study was based on the potentiation by glucose of arginine-induced insulin secretion. At low glucose, arginine caused a small rise in $[Ca^{2+}]_c$ but no

insulin secretion. Increasing the glucose concentration enhanced the rise $[Ca^{2+}]_c$, which elicited a secretory response. Importantly, with each increase in glucose concentration, the relative increase in secretion was much more marked than the corresponding increase in $[Ca^{2+}]_c$ (Ishiyama et al. 2005). Glucose thus produces signals that act distally to the Ca^{2+} signal to increase the secretory response to arginine.

In my research project, I used yet another approach comparing the effects of high glucose to those of high tolbutamide on $[Ca^{2+}]_c$ and insulin secretion. Results using this approach will be extensively described later.

2.6-Signals of amplification

Unlike the triggering pathway for which the second messenger has been almost undoubtedly identified to be the ATP/ADP ratio, the nature of the metabolism-derived messenger(s) that mediate the amplifying pathway remains uncertain. Several hypotheses have been proposed to explain the mechanisms of metabolic amplification, and a number of these have already been refuted (Sato and Henquin 1998) while others remain a subject of investigation. I briefly describe here some of the most pertinent proposals.

2.6.1-Adenine and guanine nucleotides

I previously discussed the key role played by ATP and ADP in generating the triggering signal through their action on K-ATP channels. In addition to its effect on these proximal steps in stimulus-secretion coupling, ATP might also be involved in steps distal to the $[Ca^{2+}]_c$ rise (Henquin 2009) and thus serve as a second messenger for both triggering and amplifying pathways. Such conclusions were drawn from experiments showing correlations between the ATP/ADP ratio and glucose-induced insulin secretion (Detimary et al. 1996b). When ATP production was inhibited using mitochondrial poisons without decreasing $[Ca^{2+}]_c$, insulin secretion decreased due to the loss of a distal effect of ATP (Detimary et al. 1996b). Supporting the existence of

such distal effects of ATP, are experiments in ATP-deprived primary beta-cells where photorelease of caged ATP increased membrane capacitance in the presence of stimulatory Ca^{2+} levels (Eliasson et al. 1997). Lacking in this model are the effectors on which ATP acts to increase secretion. These effectors could be cytoskeleton-related motor proteins (kinesin, myosin) or other targets involved in the final priming and fusion steps of insulin granules (discussed in chapter IV). A previous study from our laboratory (Stiernet et al. 2006) has shown that amplification by glucose is not mediated by ATP-dependent acidification of the insulin granule lumen as suggested by others (Barg et al. 2001).

There is however no consensus that an increase in ATP/ADP underlies amplification of insulin secretion by glucose. The most recent criticism rests on a very peculiar experimental protocol. Mouse islets were initially perifused for one hour with a high concentration of sulfonylurea in the complete absence of fuels. Subsequent stimulation by high glucose hardly increased ATP/ADP and failed to amplify insulin secretion. However, another metabolized agent, ketoisocaproate did increase insulin secretion without being more effective than glucose on ATP/ADP (Urban and Panten 2005). In fact, these results show that ketoisocaproate can amplify insulin secretion without much effect on ATP/ADP, but do not disprove the role of ATP in the action of glucose (inexplicably abolished in these experiments).

While GTP and GDP have a very low affinity for K-ATP channels and are not likely to affect $[Ca^{2+}]_c$ in beta-cells, they might be involved in the amplifying pathway through activation of GTP-binding proteins (G-proteins) such as Rho, Rac1, Cdc42, Rab3A and Rab27 (Kowluru 2010). Moreover, the GTP:GDP ratio was found to increase in response to glucose in parallel with the increase of the ATP:ADP ratio and to correlate with glucose-induced insulin secretion via the amplifying pathway (Detimary et al. 1996b).

2.6.2-NADPH/NADP

There is evidence that signals other than ATP are produced by beta-cell mitochondria, which can modulate insulin secretion possibly via the amplifying

pathway. That the NADPH/NADP ratio might be one of these signals is suggested by a strong correlation between this ratio and insulin secretion (Ivarsson et al. 2005b). In the beta-cell, NADPH production is mostly mitochondrial and the export of NADPH equivalents to the cytosol can take place through three pathways: the malate and citrate pathways in humans and rat and the isocitrate pathway in human, rat and mouse islets (MacDonald et al. 2005b).

While NADPH and $NADP^+$ do not have a physiological effect on K-ATP channels, changes in the NADPH/NADP ratio can regulate voltage-dependent K^+ channels thus affecting action potentials and the triggering Ca^{2+} signal (MacDonald et al. 2003). Furthermore, NADPH seems to be involved in steps distal to the rise in $[Ca^{2+}]_c$ since it increases exocytosis assessed by membrane capacitance measurements in permeabilized beta-cells (Ivarsson et al. 2005b). The effectors involved in this pathway are unknown but might be regulators of granule movement (NEM-sensitive factor) or granule priming (glutaredoxin and thioredoxin) (MacDonald et al. 2005a). The group of Panten, who argues against the role of ATP, also refutes the role of NADPH/NADP on the basis of similar arguments: persistence of an amplification of insulin secretion by ketoisocaproate despite suppression of the normal rise in NADPH/NADP ratio by long pretreatment with a sulfonylurea in absence of any fuels (Panten and Rustenbeck 2008).

2.6.3-AMP-activated protein kinase (AMPK)

AMPK is a major cellular energy sensor and a regulator of metabolic homeostasis. This kinase is a heterotrimeric complex comprising a catalytic α subunit where the threonine phosphorylation site is located, and regulatory β and γ subunits, the latter containing the AMP binding site (Hardie et al. 2006). AMPK can be activated by two distinct signals: a Ca^{2+}-dependent pathway mediated by a calmodulin-dependent kinase kinase β (CaMKKβ) activated upon elevation of $[Ca^{2+}]_c$ and an AMP-dependent pathway mediated by the upstream serine/threonine kinase 11 (STK1) or liver kinase B1 (LKB1) (Hardie 2008) and activated when intracellular AMP increases.

The involvement of AMPK in insulin secretion has been under investigation for the past ten years. However there is still no agreement on the precise effect of AMPK activity on the secretory response of beta-cells, and results published by different groups are often contradictory. Interestingly, some (Tsuboi et al. 2003) but not all (McDonald et al. 2009) studies suggested that AMPK activity can affect insulin secretion by modifying components of the cytoskeleton or their related motor proteins. To date, no studies have directly investigated the involvement of AMPK in metabolic amplification although this hypothesis has been proposed (Rutter et al. 2003, Tsuboi et al. 2003). Key-results regarding the role of AMPK in insulin secretion and its metabolic amplification will be detailed later when I describe personal data (see chapter VI).

2.6.4-Insulin granule mobilization by the cytoskeleton

Throughout the 1970's, there was remarkable interest in the role of the beta-cell cytoskeleton in the regulation of insulin secretion. Indeed, data from several pioneer studies performed at that time showed alterations in glucose-induced insulin secretion upon treatment with microtubule or microfilament-modifying drugs. The necessity for insulin granules to travel to the membrane along microtubules and then cross a barrier of microfilaments to gain access to exocytotic sites became a common statement in many textbooks.

During the last ten years, there has been renewed interest in the beta-cell cytoskeleton and its role in insulin granule trafficking and secretion. Moreover, it has been proposed that the amplifying effect of glucose involves changes in insulin granule handling by the cytoskeleton. This hypothesis was the main question of my thesis. A detailed review of previous studies that led us to investigate this pathway can be found in chapter V.

IV. THE KINETICS OF INSULIN SECRETION

1-Peculiar biphasic kinetics of glucose-induced insulin secretion

Glucose-induced insulin secretion displays a characteristic biphasic pattern. When the glucose concentration is abruptly increased and then remains elevated, insulin secretion rapidly accelerates (1^{st} phase) before declining to a nadir and then continuing in a steady or ascending fashion (2^{nd} phase). *In vitro*, this pattern was first described by Grodsky and colleagues using the isolated and perfused rat pancreas (Curry et al. 1968). Not long afterwards, a similar pattern was observed with perifused isolated rat islets (Lacy et al. 1972). *In vivo*, a biphasic increase in plasma insulin levels was described in experiments where glucose was administered intravenously to healthy subjects (Cerasi et al. 1967, Porte and Pupo 1969).

1.1-Biphasic insulin secretion *in vitro*

In vitro, the first phase of glucose-induced insulin secretion is defined as the initial burst of insulin which is released in the first 5-10 minutes following beta-cell exposure to a rapid increase in glucose. Following this initial burst, insulin secretion continues as long as the stimulus (elevated glucose) persists, but the kinetics of this second phase is species-dependent. It increases with time in experiments using the perfused rat pancreas or isolated rat islets (Fig. 6A) and is flat in the perfused mouse pancreas or isolated mouse islets (Fig. 6B) and in human islets (Fig. 6C).

1.2-Biphasic insulin secretion *in vivo*

During hyperglycemic clamps, plasma insulin levels increase with a biphasic pattern in humans (Fig. 6D) and mice (Henquin et al 2006a, Nunemaker et al. 2006). It is noteworthy that in both species, the second phase is ascending although the rate of insulin secretion by isolated islets studied *in vitro* is not (Fig. 6B and C).

Studying the kinetics of insulin secretion *in vivo* is not easy because measurements of plasma insulin levels in a peripheral vein reflect not only insulin

secretion but also insulin distribution, degradation and accumulation. Moreover, about 50% of secreted insulin is extracted by the liver before entering the peripheral circulation. Nowadays, this difficulty can partly be overcome by measuring C-peptide that is equimolarly co-secreted with insulin but does not undergo hepatic extraction (Polonsky 1994). In addition, deconvolution equations are used to calculate the secretion rate from changes in peripheral concentrations (Caumo and Luzi 2004, Cobelli et al 2007). In heroic times, however, portal vein catheterization established the biphasic kinetics of insulin secretion in normal human subjects (Blackard and Nelson 1970).

In vivo, a true first phase of insulin secretion is induced when the concentration of glucose is abruptly increased by intravenous injection of glucose. When glucose is given as a bolus, only a first phase is produced. To induce a second phase, a hyperglycemic clamp must be performed. This rapid increase in glucose differs from the more gradual changes observed when an equivalent amount of glucose is administered orally (Caumo and Luzi 2004). Under physiological conditions (eg after meals), insulin secretion increases monotonically and no well-defined first phase is observed. It is, however, widely admitted that the first phase insulin response to an intravenous glucose challenge and the early response to oral glucose rely on common beta-cell mechanisms (Caumo and Luzi 2004, Mari and Ferrannini 2008). One can thus view the first phase of insulin secretion as a magnification of the ability of beta-cells to deliver a quick response to gradually increasing glucose levels. This quick response is crucial for optimizing the effects of insulin. The early insulin response following glucose ingestion plays an important role in the maintenance of postprandial glucose homeostasis mainly through rapid inhibition of endogenous glucose production in the liver.

1.3-Altered biphasic insulin secretion in type 2 diabetes

Patients with type 2 diabetes display abnormalities in early insulin secretion in response to glucose (Pratley and Weyer 2001). Intravenous glucose tolerance tests, which elicit biphasic insulin secretion in normal subjects, reveal two disturbances in

type 2 diabetic subjects: the first phase is very low, often absent, and the second phase is lower that in non-diabetic controls at identical plasma glucose (van Haeften 2002). In subjects with impaired glucose tolerance and impaired fasting glycemia, the first phase of insulin secretion in response to a hyperglycemic clamp is blunted while the second phase is not significantly different from that of subjects with normal glucose tolerance (van Haeften et al. 2002). This selective loss of the first phase, i.e. the capacity of beta cells to quickly adjust their secretory rate in response to glucose, is an early sign of beta-cell alteration which can lead to the development of type 2 diabetes.

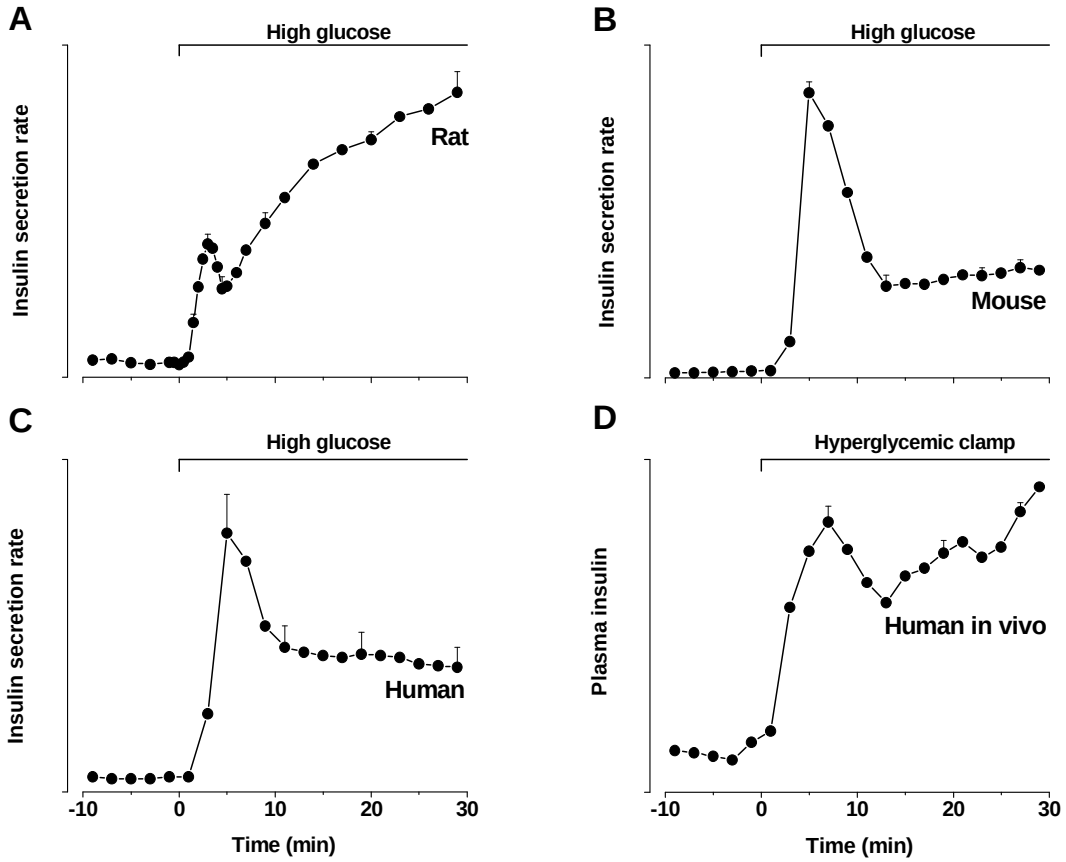

Fig. 6: Glucose-induced biphasic insulin secretion.
A, B and C: *In vitro* insulin secretion by isolated rat, mouse and human islets stimulated by an increase of the glucose concentration in the perifusion medium. D: Plasma insulin levels in healthy human subjects during a hyperglycemic clamp. A is taken from Henquin et al. 1980 and C from Henquin et al. 2006. D is adapted from Caumo and Luzi 2004. B shows personal results.

1.4-The insulin pulsatility

The temporal resolution of the technique used to monitor insulin secretion also has a significant impact. Our description of ascending or flat patterns of insulin secretion during the second phase is an oversimplification. Simultaneous measurements of insulin secretion and $[Ca^{2+}]_c$ in the same islet have shown that each glucose-induced $[Ca^{2+}]_c$ oscillation is accompanied by a synchronous oscillation of insulin secretion (Fig. 7). Detection of this pulsatility requires a much higher sampling rate than in most experiments. Moreover, this pulsatile pattern is masked by pooling data from several islets which oscillate at different frequencies (Henquin 2009). Pulsatility of insulin secretion can be revealed by deconvolution of results obtained with groups of perifused islets (Ritzel et al. 2003).

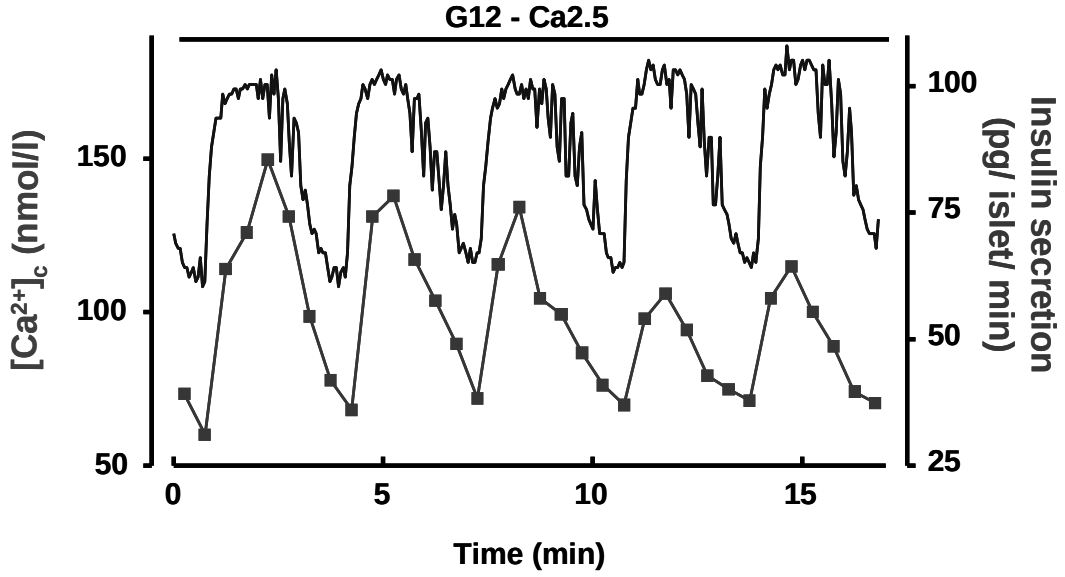

Fig. 7: Synchronous $[Ca^{2+}]_c$ and insulin secretion oscillations in response to glucose.
In response to 12 mM glucose (G12) an individual mouse islet displays oscillations of $[Ca^{2+}]_c$ (top trace) that entrain synchronous pulsatile insulin secretion (bottom trace). Frequent sampling of effluent fractions in insulin secretion experiments allows detection of the oscillatory pattern of the secretory response to glucose. (results obtained by Magalie Ravier).

As mentioned before, pulsatile insulin secretion is observed *in vitro* with the intact perfused pancreas (Lefebvre et al. 1987). How such a synchronization between islets is achieved is not clearly understood, but intrapancreatic neurons have been proposed as "external synchronizing pacemakers" (Fridlyand et al. 2010, Bertram et

al. 2010). Oscillations in plasma insulin levels also exist apparently independent of oscillations in plasma glucose. Impairment of this pulsatility in the post-absorptive state has also been reported in glucose-intolerant and type 2 diabetic patients (Caumo and Luzi 2004). Irregularity or complete loss of oscillations was observed in diabetic patients and their first degree relatives (Lefebvre et al. 1987).

2-Models of biphasic insulin secretion

Early hypotheses have variably attributed biphasic insulin secretion to intra-islet beta-cell heterogeneity with different populations of beta-cells preferentially secreting during the first or second phase, to selective secretion of newly synthesized granules during the second phase or to a negative feedback exerted by insulin on its own secretion via beta-cell insulin receptors (Curry et al. 1968, Grodsky et al. 1969). These hypotheses have been refuted and recently replaced by two major, non exclusive models: the signal-limited model and the storage limited model (review in Henquin 2009).

2.1 -Signal-limited model

This model proposes that biphasic insulin release results from the dynamic interaction between stimulatory and inhibitory events initiated by glucose. These include time-dependent potentiation and time-dependent inhibition signals which together shape the secretory response. Following triggering of the first phase, a time-dependent inhibition is generated which decreases secretion until a nadir is reached; thereafter a time-dependent potentiation appears which promotes second phase secretion. The nature of these putative potentiating and inhibitory signals has never been well defined, although some authors suggested that the potentiation involves PKC activation (Nesher and Cerasi 2002).

Alternatively, biphasic insulin secretion could result from the biphasic time course of a single, positive signal (Henquin 2009). In this case, the Ca^{2+} signal seems a very plausible candidate (Henquin et al. 2006b) since it displays a biphasic pattern as we will see below.

2.2-Storage limited model

This model proposes that biphasic insulin secretion results from the exocytosis of insulin granules located in geographically and/or functionally distinct pools. This concept was first put forward by Grodsky (1972) on the basis of a mathematical analysis of the pattern of insulin secretion observed with the perfused rat pancreas. More direct support for the heterogeneity of insulin granules within a single beta cell was obtained much more recently (see below, paragraph 5). In brief, upon glucose stimulation, an immediately releasable pool (IRP) of granules undergoes exocytosis in response to depolarization and increased Ca^{2+} entry. At the nadir after the first phase, IRP is depleted; this slows down granule release. New granules must then be converted to the IR state to sustain second-phase secretion (Straub and Sharp 2004). This replenishment of the IRP may be achieved by granules already attached to the membrane (docked) or mobilized from a reserve pool.

3-Biphasic kinetics of glucose-induced membrane potential and $[Ca^{2+}]_c$ changes

An important common feature of both first and second phases is their dependency on extracellular Ca^{2+}. In a Ca^{2+}-free medium, glucose fails to induce either of the two phases and no secretory response is observed (Charles et al. 1975, Henquin 1978).

As shown in figure 2, glucose-induced depolarization and electrical activity display a biphasic time course in mouse beta-cells. The first phase usually starts one minute after the change in glucose concentration (Gilon and Henquin 1992) and is characterized by a gradual then rapid depolarization, followed by a train of action potentials that appear as spikes superimposed on a plateau of depolarization. This first phase is terminated by the stopping of spike activity and partial repolarization of the membrane. The second phase then starts with the characteristic oscillatory pattern described earlier.

The changes in $[Ca^{2+}]_c$ mirror the pattern of membrane potential changes induced by glucose, with one exception. During the initial slow depolarization

$[Ca^{2+}]_c$ decreases due to Ca^{2+} pumping into the ER. Then, during the first phase, $[Ca^{2+}]_c$ increases simultaneously in all beta-cells of the islet. Notable also is the synchronization between different islets in an *in vitro* setting, and probably also in a whole pancreas when it comes to the triggering of the $[Ca^{2+}]_c$ rise during the first phase. Therefore, averaging the responses from several islets results in a well-defined first phase of $[Ca^{2+}]_c$ (Fig. 3C) as if the sudden increase in glucose concentration acts as an extrinsic synchronizer (Henquin 2009).

During the second phase, the membrane potential and $[Ca^{2+}]_c$ oscillations are synchronous between beta-cells of each single islet (Meissner 1976, Gilon and Henquin 1992). This synchronization is due to the electrical coupling of beta-cells via gap junctions made of connexin 36 (Ravier et al. 2005). As a result, $[Ca^{2+}]_c$ oscillations lead to insulin pulsatility at the islet level (Fig. 7). However, even between islets isolated from the same mouse, $[Ca^{2+}]_c$ oscillations in response to glucose stimulation are not synchronous (Fig. 3A), so that averaging $[Ca^{2+}]_c$ measurements from different islets markedly attenuates the oscillatory pattern (Fig. 3B) (Henquin 2009). I will not expand on this issue because resolution of the pulsatility of insulin secretion was never attempted in my experiments.

It has been suggested that different types of Ca^{2+} channels are involved in each of the two phases of insulin secretion. Pharmacological blockade of R-type Ca^{2+} channels or their knock-out in mice caused a 20% decrease of the secretory response specifically during the second phase in parallel with an 18% reduction of oscillatory $[Ca^{2+}]_c$ (Jing et al. 2005). This view is however at odds with observations that specific L-type channel blockers completely inhibit both phases of the secretory response and that the Ca^{2+} current reflects activation of L-type channels in mouse beta-cells (Rorsman et al. 2011).

The biphasic patterns of the changes in triggering Ca^{2+} and of insulin secretion are thus very similar, similar enough to warrant the reasonable suggestion of a causal relationship. This similarity holds for different glucose concentrations. Thus, the duration of first phase rise in $[Ca^{2+}]_c$ increases with glucose concentrations, as does the duration of first phase insulin secretion (Henquin et al. 2006b). Does this rule out

the existence of functionally distinct pools of insulin granules? I will address the question by comparing insulin secretion and $[Ca^{2+}]_c$ changes in response to other stimuli and by discussing the evidence supporting the participation of granular pools below.

4-The kinetics of insulin secretion and $[Ca^{2+}]_c$ changes induced by other stimuli

There is general consensus that, in addition to glucose, metabolized substrates such as amino acids induce biphasic insulin secretion in rodent and human islets (Henquin et al. 2006a). Conversely, it has often been stated that beta-cell depolarization with non-metabolized agents such as sulfonylureas or KCl elicits just a rapid and short-lived burst of insulin secretion (1^{st} phase) and no second phase that was considered as a hallmark of the response to fuels. This is not simple. The pattern is much dependent on the experimental conditions (type of preparation, species, concentrations of tested agents, and presence or not of a low glucose concentration). Because such a discussion is not relevant to my own studies, I will restrict the following paragraphs to the effects of tolbutamide and KCl in perifused mouse islets, the preparation that I have used.

4.1-KCl

Increasing the concentration of extracellular K^+ shifts the equilibrium potential of K^+ towards more positive values. Beta-cell stimulation with 30 mM KCl produces a continuous depolarization of the plasma membrane (Gilon and Henquin 1992) characterized by the absence of action potentials. In response to KCl stimulation, $[Ca^{2+}]_c$ rapidly increases to a peak lasting no more than 1-2 min, followed by a sustained slightly increasing plateau (Fig. 8C). In the absence of glucose, K^+-induced insulin secretion is transient (Henquin 2000) and monophasic, but becomes biphasic in the presence of as little as 1 mM glucose (Fig 4B). The second phase is even increasing in the presence of 15 mM glucose (Fig. 8F).

4.2-Tolbutamide

Sulfonylureas such as tolbutamide stabilize the closed state of K-ATP channels by binding to one of the four SUR1 subunits and thereby cause beta-cell depolarization (Drews et al. 2010). The ionic mechanism is thus the same as for glucose but does not involve changes in metabolism. In all my experiments tolbutamide was used at 500 µM, which is a maximally effective concentration selected to close all K-ATP channels (Henquin 2000). When added to a perifusion medium containing glucose at a non-stimulatory concentration (3 mM), 500 µM tolbutamide caused a rapid increase in $[Ca^{2+}]_c$ followed by a rather sustained plateau (Fig. 8B). The resulting stimulation of insulin secretion was characterized by a rapid first phase followed by a stable plateau, higher than basal levels of secretion. The secretory response to tolbutamide is thus biphasic in the presence of a low glucose concentration (Fig. 8E).

4.3-KCl and tolbutamide as tools to study the kinetics of secretion

KCl has often been used to depolarize beta-cells and stimulate Ca^{2+} influx through voltage-dependent Ca^{2+} channels in the perspective to mimic glucose effects on first phase insulin secretion independently of metabolic changes. This approach has been criticized (Hatlapatka et al. 2009) for two reasons: the mechanisms of depolarization are different and the increase in $[Ca^{2+}]_c$ produced by KCl is usually greater than that produced by glucose. It is therefore plausible that high KCl stimulation elicits effects that do not occur upon physiological (glucose) closure of K-ATP channels by glucose (Hatlapatka et al. 2009). In my own studies, I have therefore privileged the comparison of glucose and tolbutamide effects. However, it is obvious that the kinetics of $[Ca^{2+}]_c$ and insulin secretion changes are not identical during either KCl or tolbutamide stimulation. They are dissimilar enough to suggest that triggering Ca^{2+} is not the sole determinant of insulin secretion. Notably, it is striking that a short-lived initial peak of $[Ca^{2+}]_c$ triggered a much longer first phase of secretion, which was actually as long as that produced by glucose (Fig. 8D). Ca^{2+} and

insulin responses were also dissociated during second phase. These discrepancies are at least compatible with the existence of granular pools, which I will now discuss.

5-The kinetics of insulin secretion in single beta-cells: role of granular pools

In vitro, the kinetics of insulin secretion is most often monitored using the intact, perfused pancreas or groups of isolated and perifused islets. A few studies show that the biphasic pattern is also observed in single mouse islets stimulated by glucose (Henquin et al. 2002).

It is possible to monitor insulin secretion at the single beta-cell level. A drawback of this experimental model is that single primary beta-cells secrete considerably less insulin than beta-cells within intact islets, except when they are boosted by cAMP. Amperometric techniques are available but lack the necessary resolution to identify the time-course of the responses evoked by glucose (Huang et al. 1995). Exocytotic events can be measured as changes in membrane capacitance as explained earlier. However, the response to glucose alone is beyond resolution power. Photorelease of caged Ca^{2+} or electrical depolarization in patch-clamped isolated mouse beta-cells evokes an increase in capacitance with an initial rapid, followed by a slower, sustained component, which can be derived into a biphasic change in the rate of exocytosis (Eliasson et al. 1997, Barg et al. 2001). These experiments combined with morphological studies of beta-cells (Olofsson et al. 2002) provided the basis for the subdivision of insulin granules in distinct pools.

Two notes of caution however seem relevant. First, these electrophysiological measurements are performed over considerably shorter periods of time (1-2 seconds) than traditional assays with islets (5-10 min for first phase only). Second, when capacitance measurements are performed in beta-cells within an intact islet, depolarization-evoked exocytosis is surprisingly monophasic and occurs at rates much lower than those reported in isolated cells (Gopel et al. 2004). It could be argued that these measurements are biased by the fact they report exocytosis only in

superficial beta-cells, but a linear relation between stimulation and exocytosis was also found in beta-cells located in the islet centre (Speir and Rupnik 2003).

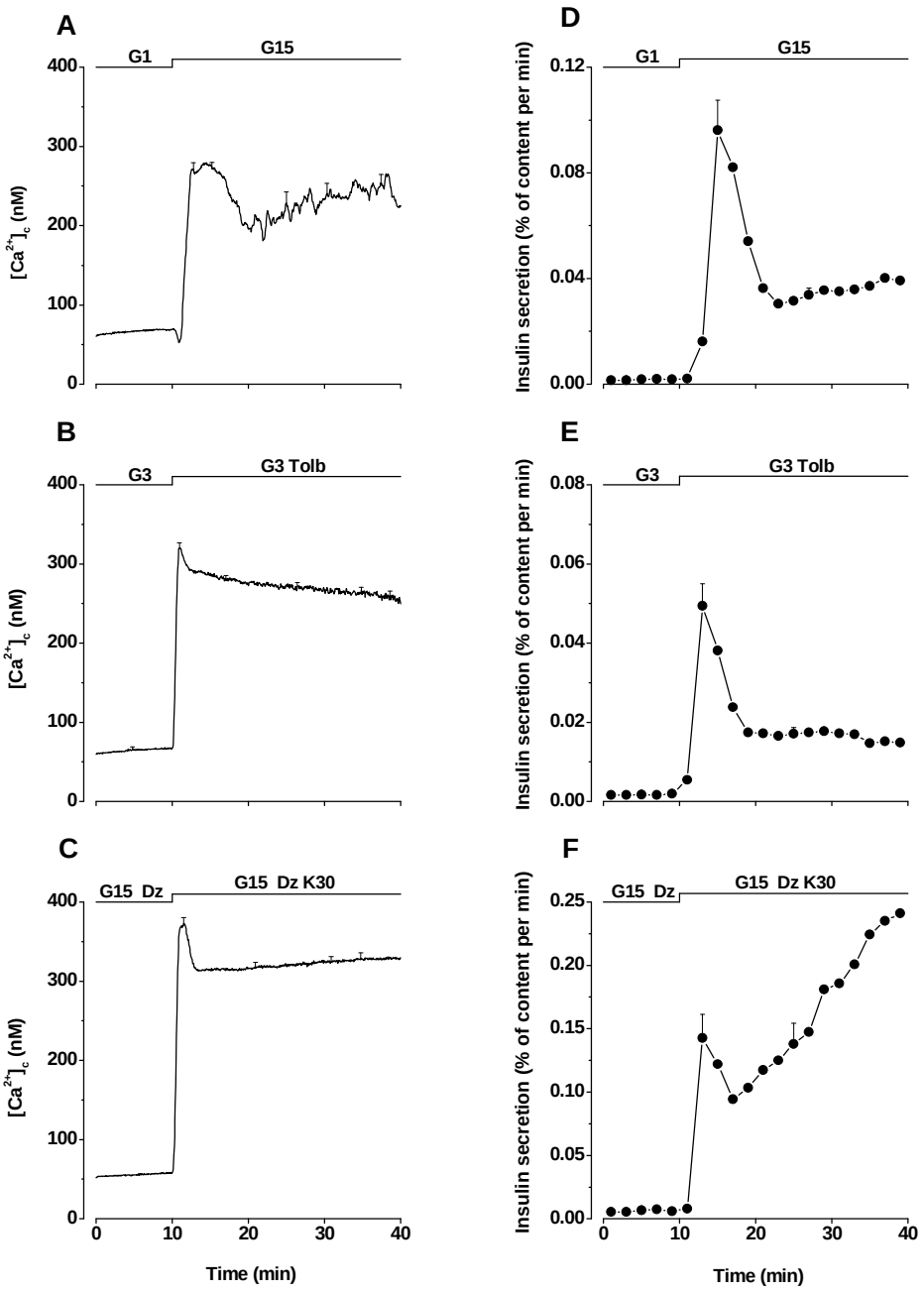

Fig. 8: Comparison of $[Ca^{2+}]_c$ (A, B and C) and insulin secretion (D, E and F) changes induced by glucose (A and D), tolbutamide (B and E) and KCl (C and F) in mouse islets. Islets were stimulated with 15 mM glucose (G15), 500 µM tolbutamide (Tolb) in the presence of 3 mM glucose (G3) or 30 mM KCl (K30) in the presence of 15 mM glucose and 100 µM diazoxide (Dz). The results are the means ± SEM of 9-11 separate experiments (personal results).

The technique of TIRFM (total internal reflection fluorescence microscopy) has also been used to study the dynamics of insulin granules and their behavior during biphasic insulin secretion. This imaging method permits fluorescence excitation and visualization of a narrow region (within 100 nm) just beneath the plasma membrane (Axelrod 2001) and observation of single secretory granules undergoing exocytosis (Ohara-Imaizumi et al. 2002). It was thus found that exocytosis occurs in a biphasic fashion with some granules preferentially secreted during the first phase while others required more than the triggering Ca^{2+} underwent exocytosis during the second phase (Ohara-Imaizumi et al. 2002).

6-Insulin granule pools

Insulin is packaged into large dense core vesicles (or insulin granules with a diameter of 300-350 nm) stored in the beta-cell cytosol. In addition to insulin, these secretory vesicles contain C-peptide, several enzymes, Zn^{2+}, Ca^{2+}, ATP, dopamine, serotonin and glutamate (Hutton et al. 1983). In this section I will describe the different pools of granules thought to exist in beta-cells and the functional significance of these populations.

Morphometric studies of mouse and rat beta cells by electron microscopy have yielded consistent estimates of insulin granule numbers: 9000-13000 per beta-cell (Dean 1973, Olofsson et al. 2002, Straub et al. 2004). In the rest of my thesis, I will use a value of 10000 for the total number of granules. On morphological bases, one distinguishes granules in the cell interior (the large reserve pool) and granules close to the plasma membrane (the docked pool) can be distinguished. On a functional basis, these granules can also be subdivided according to their readiness for exocytosis. Figure 9 schematizes the insulin granule pools and some of the cellular components involved in the different steps of secretory vesicle exocytosis.

6.1-The reserve pool

The reserve pool comprises more than 90% of the granules in a normal beta cell. Of these ~9000 granules, ~7500 are located at variable distances >200nm away from the plasma membrane and constitute the true reserve pool, whereas ~1500 are situated <200 nm from the surface and said to be “almost docked” (Olofsson et al. 2002). All these granules may be of different ages. Interestingly, newly synthesized granules seem to be secreted preferentially to older ones (Gold et al. 1982, Halban 1982) but neither the significance nor the mechanisms of the phenomenon have been explained.

6.2-The docked pool

Docked granules are characterized by their close apposition to the plasma membrane. The number of these granules is estimated to be around 600 in mouse beta-cells (Olofsson et al. 2002) and 700 in rat beta-cells (Straub et al. 2004). From a molecular point of view, docking ensures that secretory granules are anchored to the plasma membrane (Becherer and Rettig 2006) making them seemingly poised for exocytosis upon stimulation.

The proteins involved in granule docking have not been all identified. The classic notion that docking occurs through pairing of the plasma membrane with vesicle SNARE complexes is disputed. Granule docking defects were found in syntaxin-1a and SNAP-25 null cells but no defects were found in synaptobrevin2 null cells (Izumi 2011). Among the other candidate molecules for docking is Munc18-1 through its interaction with the Rab27A effector granuphilin (Izumi et al. 2007).

Another group has found that interaction of Rab3 with its effector Rim2α is required for docking (Yasuda et al. 2010). The lack of a consensus model for granule docking stems from the difficulty in measuring docking independently of the other steps that lead to exocytosis, for instance granule priming. Some effectors might be involved in both processes which complicates the definition of their role in each of these steps.

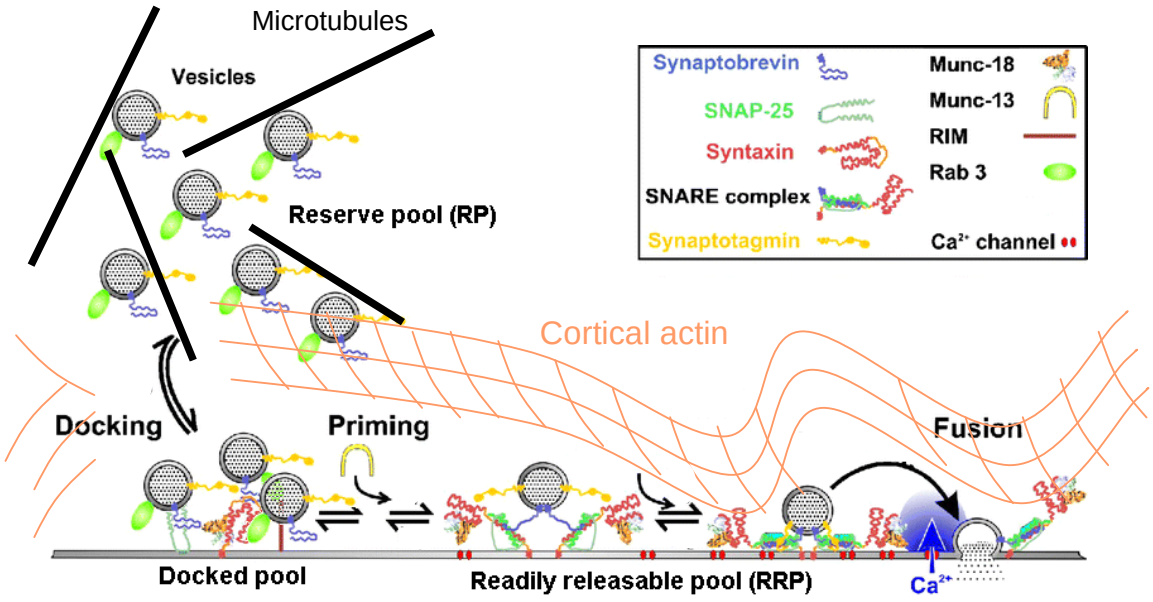

Fig. 9: Schematic representation of the classical granule pools theory.
Insulin granules are thought to move along microtubules from the reserve pool to the vicinity of the plasma membrane. The cortical actin barrier is subject to glucose-induced reorganization giving granules access to the docking sites. Different molecules possibly involved in docking (synaptobrevin, syntaxin, Rab, RIM, Munc-18) are represented. Priming probably involves SNARE complex reorganization by Munc-13 and renders the granules readily releasable. Synaptotagmin functions as a calcium sensor and initiates granule fusion to the plasma membrane in response to calcium entry (adapted from Becherer and Rettig 2006).

6.3-The immediately releasable pool

Studies using TIRF microscopy, which allows the imaging of vesicles in close proximity to the plasma membrane (<100-200 nm), showed that only a small subset of the docked granules are releasable (Izumi et al. 2007). This confirms the conclusions of electrophysiological studies which estimated their number between 50-100 per beta-cell (Rorsman and Renstrom 2003). These granules have gone through a series of ATP-requiring maturation steps to acquire full release competence: the priming process. They are thus functionally, not morphologically, distinct from the bulk of the 600 docked granules. In experiments using a beta-cell line, docked granules were identified by co-immunoprecipitation of a v-SNARE protein (VAMP2) with antisera against a t-SNARE protein (syntaxin), which indicates stable formation of the complex (Daniel et al. 1999). Short stimulation of the cells with glucose or KCl decreased the size of this pool. KCl stimulation of

mouse islets also decreased the number of morphologically docked granules (Olofsson et al. 2002). These observations led to the suggestion that a subset of the docked pool corresponds to the readily releasable pool responsible for first phase.

Several mechanisms have been suggested to underlie granule priming. One popular view attributes it to the disassembly of the SNARE complex followed by its reorganization, catalyzed by NSF and SNAP proteins, to allow increased membrane contact (Klenchin and Martin 2000). Protein or lipid kinases, present on dense core vesicles as a membrane-bound enzyme or as a soluble cytosolic factor, are also essential for granule priming in chromaffin and pancreatic beta-cells (Klenchin and Martin 2000, Olsen et al. 2003). Phosphoinositide kinases have recently attracted much interest (Kwan and Gaisano 2007). Phosphoinositides are known to bind synaptotagmin and rabphilin among others but whether their interaction with these exocytotic proteins is involved in granule priming is not fully elucidated. Munc proteins are required for release of the RRP granules during both phases of insulin secretion (Kwan and Gaisano 2007). Munc18 binds syntaxin1 in its closed form and, in concert with Munc13, might regulate SNARE complex formation and final fusion steps (Kwan and Gaisano 2009).

I have already mentioned the proposals that 1) insulin granule priming requires intragranular acidification by a H^+-ATPase, and 2) that this acidification corresponds to the amplifying effect of glucose (Barg et al. 2001). A previous study from our laboratory has shown that the acidic pH of granules is important for insulin secretion, which would be compatible with the first proposal but refuted the second (Stiernet et al. 2006).

Based on this distribution of insulin granules, a model emerged in which granules need to be docked to the plasma membrane before acquiring release competence and undergoing the final fusion step. According to this popular model, a subset of docked granules are primed and thus form the immediately releasable pool (IRP) that undergoes fast exocytosis during the first phase response to glucose (Rorsman and Renstrom 2003). Insulin secretion then decelerates upon emptying of this IRP. The rate of secretion during the second phase is determined by the rate of

granule recruitment from the reserve pool and their docking or by the rate of priming of previously docked granules (Bratanova-Tochkova et al. 2002).

6.4-The popular model needs revision

Analysis of insulin granule dynamics using TIRFM microscopy revealed novel aspects of insulin granule exocytosis.

One study suggested that the two phases of release differ in their requisite SNARE protein isoforms. First-phase insulin release appears to utilize syntaxin 1A, syntaxin 4, SNAP25 or SNAP23, and the v-SNARE VAMP2, whereas second-phase secretion is managed by Syntaxin 4, SNAP25 or SNAP23, and VAMP2, but specifically not Syntaxin 1A (Ohara-Imaizumi et al. 2007**).**

In other studies, granules were found to bypass the docking step and immediately fuse to the plasma membrane (Izumi et al. 2007) Moreover, granules behaving this way accounted for most of the insulin released during both phases of glucose-induced insulin secretion whereas K^+-induced insulin granule exocytosis resulted from the release of docked granules (Seino et al. 2011). This was explained by the fact that K^+ stimulation induces supraphysiologic levels of $[Ca^{2+}]_c$ which force the exocytosis of docked granules. The rise in $[Ca^{2+}]_c$ brought about by glucose is smaller (Seino et al. 2011) and seems to act preferentially on a subset of highly Ca^{2+} sensitive granules not located in close proximity of VDCCs (Pedersen and Sherman 2009).

Using the same TIRFM imaging technique, another group found that sulfonylurea also caused rapid fusion mostly from newcomer insulin granules rather than previously docked or “resident” granules (Nagamatsu et al. 2006). Collectively, these recent data suggest that docking is a braking state that prevents the fusion of granules to the plasma membrane (Seino et al. 2011). Indeed, mice bearing a knock-out of granuphilin which is indispensable for insulin granule docking, exhibit enhanced insulin secretion despite a decrease in the number of docked granules (Izumi 2011). Finally, this revised model implies that priming, i.e. acquirement of full release competence, can involve both docked and undocked granules. The latter

subset of granules which appear in the vicinity of the plasma membrane within just 50-300 milliseconds prior to the fusion event (Izumi 2011) are also referred to as almost docked or highly Ca^{2+}-sensitive granules to differentiate them from the large reserve pool.

V. THE BETA-CELL CYTOSKELETON AND INSULIN SECRETION

Like most other cells, beta-cells contain a cytoskeletal structure primarily made up of microtubules and microfilaments, and of intermediate structures and proteins that anchor or move along them.

1-Microtubules

1.1-Microtubule structure and dynamics

Microtubules are long, hollow structures with 5 nm walls surrounding a cavity 15 nm in diameter. They are composed of two globular protein subunits, α and β tubulin, 55 kDa each. The two subunits form heterodimers which aggregate into long tubes made up of stacked rings, with each ring usually containing 13 subunits or protofilaments.

Microtubules undergo continuous assembly and disassembly. The end where assembly predominates, called the *plus* end, usually faces the plasma membrane, and the end where disassembly predominates is called the *minus* end (Loubéry and Coudrier 2008). They are maintained in a dynamic equilibrium of soluble subunits and polymerized microtubules, the ratio of which may be regulated by a number of physiological factors.

Every α-β tubulin heterodimer is bound to two molecules of GTP. The α subunit tightly binds one GTP molecule that cannot be removed, whereas the β subunit binds the other GTP molecule that is freely exchangeable (Jordan et al. 1998) and becomes hydrolyzed to GDP and P_i as tubulin polymerizes onto a growing microtubule end (Wilson et al. 1999b). When a GDP-bound dimer is released upon microtubule disassembly, it is rendered assembly-competent again by exchanging the β subunit-bound GDP for GTP (Calligaris et al. 2010).

Microtubule-associated proteins (MAPs) are 200 kDa proteins involved in the formation and stabilization of microtubules, ‘’protecting’’ them from the depolymerizing effect of low temperature and Ca^{2+} ions (Jordan et al. 1998). These MAPs also mediate microtubule cross-linking and their interaction with other proteins. Several MAPs are specific to neural tissues while others, such as MAP4, have a ubiquitous distribution (Mandelkow and Mandelkow 1995). These proteins are activated by phosphorylation, probably by microtubule affinity-regulating kinases (MARKs) (Drewes et al. 1998), and are involved in regulating microtubule dynamics.

Microtubules are usually organized in a radial polarized network. They form the spindle which moves the chromosomes during mitosis. They also provide tracks for the transport of vesicles, secretory granules and mitochondria from one part of the cell to another. The *minus*-end-directed motor proteins from the *dynein* family are implicated in long-range movements towards the cell interior while the *plus*-end motors from the *kinesin* family mediate long-range movement towards the cell surface (Loubéry and Coudrier 2008).

1.2-Microtubule-interfering drugs

A number of natural and synthetic products bind to tubulin and are often used as mitotic spindle poisons in the treatment of certain leukemias and solid tumors. The ability of these drugs to modify tubulin dynamics and to alter its polymerization state makes them useful tools in studying the role of microtubules in cellular functions such as secretory granule transport in regulated exocytosis. These microtubule-targeting agents can bind to different sites on the tubulin molecule and alter microtubule stability in different ways.

• *Colchicine* has been very instrumental for elucidating the properties and functions of tubulin and microtubules (Wilson et al. 1999b). Colchicine irreversibly binds to a high-affinity site on the tubulin heterodimer, alters its structure and hinders tubulin assembly, thus inhibiting microtubule polymerization.

• *Nocodazole* binds to the same site as colchicine and also inhibits tubulin assembly and causes loss of microtubules.

• Vinca alakaloids such as *vincristine* and *vinblastine* induce destabilization of polymerized tubulin by reversibly binding to a site localized on β-tubulin and prevent polymerization by blocking the region involved in heterodimer attachment (Jordan et al. 1998).

• *Taxol* (paclitaxel), unlike the other molecules, acts as a microtubule stabilizer by binding to the inner microtubule surface, stimulating microtubule polymerization and reducing tubulin subunit concentrations at equilibrium to near zero (Wilson et al. 1999b).

• *Deuterium oxide* (heavy water) overstabilizes microtubular structures and causes an increase in the number of microtubules in treated cells. However, D_2O is no longer used to stabilize microtubules because of off-target effects such as inhibition of glucose transport and mitochondrial respiration in islets (Beckman and Holze 1980).

2-Microfilaments

2.1-Microfilament structure and dynamics

Microfilaments are long solid fibers 4-6 nm in diameter. They are made up of actin, a 42 kDa protein present in all cell types. Globular actin molecules (G-actin) polymerize *in vivo* to form long filamentous chains (F-actin) known as microfilaments.

Globular actin binds an ATP molecule within the cleft located between the two lobes of the protein (Saarikangas et al. 2010). Under physiological conditions, actin monomers spontaneously polymerize into long, stable filaments with a helical arrangement of subunits. Under steady-state conditions, the net addition of subunits to actin filaments occurs at the “barbed end” or *plus* end. At the “pointed end” or *minus* end of the filament, ATP hydrolysis into ADP enables filament disassembly by regulatory proteins (Pollard and Cooper 2009).

Several accessory proteins known as actin-binding proteins (ABPs) serve to initiate polymerization (Arp2/3 complex), to regulate assembly and turnover of actin filaments (gelsolin, capping proteins) and to crosslink filaments into networks or

bundles (α-actinin, fascin). ERM proteins (Ezrin/Radixin/Moesin) are widely expressed scaffold proteins often involved in membrane-actin binding (Revenu et al. 2004). The most known regulators of actin-binding proteins are the Rho-family small GTPases, which include RhoA, Rac1 and Cdc42 (Kowluru 2010).

Microfilaments are much less rigid than microtubules. However, the presence of high concentrations of crosslinkers that bind to actin filaments promote the assembly of highly organized structures including isotropic networks, bundled networks and branched networks which form stress fibers, cortical networks under the plasma membrane and peri-organellar structures, respectively (Fletcher and Mullins 2010). Networks of cortical actin filaments provide tracks for short-range movements driven by motor proteins of the *myosin* family.

2.2-Microfilament-interfering drugs

Natural substances can bind actin and alter the dynamics of its polymerization. Among these, cytochalasins were the first to be used to investigate the involvement of actin microfilaments in cellular functions.

• *Cytochalasins* bind and block the barbed end, like a capping protein, and cause the shortening of actin filaments (Cooper 1987).

• *Latrunculins* are more specific inhibitors of actin polymerization. They probably bind to the nucleotide (ATP) binding cleft (Yarmola et al. 2000) thus sequestrating monomeric actin and hindering its polymerization (Cooper 1987). *Phalloidin* differs from cytochalasins and latrunculins in that it binds to actin filaments much more tightly than to actin monomers and, therefore, prevents actin depolymerization (Cooper 1987).

• *Jasplakinolide* shares the same binding site as phalloidin but is an even more efficient stabilizer of F-actin by decreasing the rate of dissociation of actin oligomers (Bubb et al. 1994).

3-Intermediate filaments

Intermediate filaments comprise a variety of tissue-specific proteins assembled into 10 nm-wide structures and subdivided into 5 classes according to their expression profile (Oshima 2007). These filaments are the least stiff of the three types of cytoskeletal polymers. In contrast to microtubules and microfilaments, which are made of globular proteins with nucleotide-binding and hydrolyzing activity, intermediate filaments consist of filamentous protein subunits with no enzymatic activity (Chang and Goldman 2004). Their assembly starts by the aggregation of tetramers into unit-length filaments, followed by the formation of packed filaments that compact as they elongate and mature (Chang and Goldman 2004). They can be crosslinked to each other as well as to actin filaments and microtubules by plectins. Such interactions with other components of the cytoskeleton render intermediate filaments sensitive to reorganization and motile events occurring along microfilaments and microtubules. Intermediate filaments organize the internal tridimensional structure of the cell, anchoring organelles (vimentins) and serving as structural components of the nuclear lamina (lamin). Unlike microtubules and actin filaments, intermediate filaments are not polarized and thus do not support directional movements of molecular motors (Fletcher and Mullins 2010).

4 -The beta-cell cytoskeleton

Electron microscopic studies revealed the existence of a microtubular-microfilamentous system in beta-cells about 40 years ago (Lacy et al. 1968, Malaisse et al. 1972).

Microtubules appear as straight tubules randomly scattered in the cytoplasm of perinuclear and Golgi areas, or radiating from the center to the cell periphery (Gomez-Acebo and Hermida 1973, Devis et al. 1974, Boyd et al. 1982). They are often in close relationship to various organelles including insulin secretory granules.

However, the proportion of granules associated with microtubules is debated; it is reported to range between 1-6% in peripheral zones (Boyd et al. 1982), or not to exceed that expected on the basis of random distribution (Dean 1973, Howell and Tyhurst 1982).

Quantitatively, tubulin represents a major protein in pancreatic islets averaging 0.5% of the total protein content at a concentration of about 4 ng per islet (Pipeleers et al. 1976). The microtubule-associated protein-2 (MAP-2) is expressed in islets and in insulin-secreting cell lines where it stabilizes microtubules. Its phosphorylation by calmodulin-dependent protein kinase II or PKA promotes microtubule disassembly (Mohlig et al. 1997).

Quantitatively, the total actin content of isolated islets amounts to 1-2% of total islet proteins (Howell and Tyhurst 1980). Although microfilaments can be found throughout the cytoplasm of beta-cells (Gabbiani et al. 1974), they are mainly concentrated just beneath the plasma membrane, where they form a web of variable thickness (50-300 nm). This peripheral network of bundled filaments appears to be in close relation with microtubules (Van Obberghen et al. 1973, Gomez-Acebo and Hermida 1973) and supposedly serves as a barrier preventing uncontrolled access of insulin granules to the plasma membrane (Lacy and Malaisse 1973). However, in contrast with early observations (Malaisse et al. 1975), 3-D imaging revealed the existence of insulin granules in, above and below the cortical F-actin layer (Lopez et al. 2010).

Interpretation of morphological studies of the cytoskeleton, particularly of its association with insulin granules is delicate. Indeed, cell lines that contain only few granules or purportedly degranulated beta cells may not be representative of the situation of beta-cells in situ. Moreover, single primary beta-cells (obtained by islet dispersion) and cell lines adopt an abnormal elongated form during culture on a flat support, which must affect the organization of their cytoskeleton. Finally, there occurs a marked increase in the number of microtubules and the size of the cell web in monolayer cultures of endocrine pancreas cells as compared to cells in their intact tridimensional configuration (Howell and Tyhurst 1982).

5 -Beta-cell microtubules and insulin secretion

The first evidence that beta-cell microtubules could be involved in intracellular transport of insulin granules during glucose stimulation of insulin secretion dates back to the early 1970s when Malaisse and Lacy reported inhibition of insulin secretion from rat islets exposed to mitotic spindle inhibitors. The question was then heavily investigated until the mid 1980s, before a two-decade drought period. Interest resumed with studies of microtubule-related motor proteins and the hypothesis that microtubules were involved in the amplifying pathway of insulin secretion. I will now review the most relevant data that formed the starting point of my own research project.

5.1-Inhibition of insulin secretion by anti-microtubule drugs

Stabilization of microtubules by D_2O and their disruption by vincristine or colchicine were reported to inhibit glucose-induced insulin secretion from incubated rat pancreas fragments and isolated islets (Malaisse-Lagae et al. 1971) without affecting glucose-induced Ca^{2+} uptake (Malaisse et al. 1971). Using the islet perifusion technique, it was then shown that D_2O and vinblastine inhibit both phases of glucose-induced insulin secretion as well as tolbutamide-induced secretion. Colchicine, on the other hand, inhibited only the second phase of secretion (Lacy et al. 1972). Similar results were obtained with the perfused rat pancreas: vincristine inhibited both phases of the secretory response to glucose whereas the effect of colchicine was more marked on the second phase (Devis et al. 1974, Somers et al. 1974).

In all these studies, the pancreas/islets had to be treated with the drugs for more than 90 minutes to see inhibition of secretion. After shorter exposure, insulin secretion was not affected or sometimes paradoxically increased. This delay correlated with the time needed to bind tubulin and produce a visible change in microtubule number (Montague et al. 1975). One study using rat islets found no inhibitory effect of colchicine or vinblastine on glucose-induced insulin secretion (Grill and Cerasi 1977).

At that stage, the emerging concept was that microtubules were somehow involved in the secretory response of beta-cells to glucose, particularly during the late-phase of secretion (Malaisse et al. 1975). Shortly after, studies of beta-granule movement in monolayer cultures of rat islet cells showed that glucose increased the frequency of insulin granule saltatory movements and that this increase was inhibited by anti-microtubular agents such as vinblastine and D_2O (Lacy et al. 1975, Somers et al. 1979).

Despite the consistency of the results obtained by different groups, doubts were cast on the specificity of some drugs used to alter beta-cell microtubules. Heavy water (D_2O) was found to impair glucose oxidation in rat and mouse islets (Beckmann and Holze 1980) and has since been completely abandoned. Vinblastine was reported to inhibit protein synthesis (Howell and Tyhurst 1982), an effect that can affect insulin secretion within a 2-3 hour time frame as shown by more recent experiments using cycloheximide (Garcia-Barrado et al. 2001). On the other hand, the use of newer anti-microtubular molecules at least partly corroborated early results. Taxol, a promoter of microtubule polymerization, and nocodazole, a microtubule depolymerizer, both inhibited glucose-induced insulin secretion in isolated rat islets (Howell et al. 1982). The inhibitory effect of nocodazole was confirmed in hamster islet tumor cells HIT-T15, but taxol had no effect in these same cells (Farshori and Goode 1994).

To our knowledge, only one study investigated the effect of microtubule-interfering drugs on $[Ca^{2+}]_c$ changes upon beta-cell stimulation: in MIN6 cell clusters (pseudoislets), vincristine and nocodazole inhibited the secretory response to tolbutamide and glucose, and perturbed the synchronization of $[Ca^{2+}]_c$ oscillations. Strangely, the authors suggested that this alteration was due to the loss of a "synchronizing factor" co-secreted with insulin rather than to microtubule disruption or drug side effects (Squires et al. 2002).

5.2-Microtubule dynamics upon glucose stimulation

The observation that microtubule disrupters inhibit the second phase of glucose-induced secretion prompted the suggestion that insulin granule mobilization along microtubules is required for this sustained phase, and raised the hypothesis that the process is facilitated by tubulin polymerization (Malaisse 1975).

Using a tubulin binding assay, it was found that ~30% of total tubulin is in polymerized form in non-stimulated rat islets. Exposure to glucose slightly increased this percentage to ~40% indicating an increase in the number of microtubules in response to glucose (Montague et al. 1976, Pipeleers et al. 1976). These changes in tubulin polymerization temporally correlated with the biphasic pattern of the secretory response: insulin secretion and the amount of polymerized tubulin increased two minutes after glucose stimulation of rat islets, before decreasing to a nadir and then gradually increasing during the second sustained phase (McDaniel et al. 1980). What is intriguing and remains unexplained is how glucose favors microtubule formation while increasing $[Ca^{2+}]_c$ which is known to inhibit microtubule assembly.

5.3-Involvement of microtubule-related motor proteins

The studies mentioned above provided evidence that the microtubule cytoskeleton is a potential target for glucose-generated signals in pancreatic beta-cells. However, detailed insight on how microtubules actually participate in stimulus-secretion coupling was still missing. Attention was then directed toward specific microtubule related motor proteins. One of these, kinesin, a microtubule-associated ATP, has been the focus of several studies.

Kinesin, a plus end-directed microtubule motor protein that moves vesicles from the cell center toward the cell cortex, has been identified and purified from beta-cells (Balczon et al. 1992). Inhibition of kinesin expression by antisense oligonucleotides inhibited basal and glucose-induced insulin secretion from primary beta-cells (Meng et al. 1997). In line with these findings, expression of a dominant-negative form of the kinesin heavy chain, the ATP and microtubule-binding domain

of kinesin, strongly inhibited sustained (measured at t=90 min) but not acute (measured at t=20 min) glucose-induced insulin secretion in MIN6 cells. In parallel to this inhibition, ATP-dependent vesicular movements were apparently blocked by the loss of kinesin activity (Varadi et al. 2002).

Another study suggested that glucose regulates kinesin activity not only by providing energy in the form of ATP but also by means of $[Ca^{2+}]_c$ changes (Donelan et al. 2002). Kinesin heavy chain was found to be dephosphorylated by a protein phosphatase under conditions of elevated $[Ca^{2+}]_c$. Moreover, inhibition of this dephosphorylation reduced the second phase of insulin secretion in primary rat islets and INS-1 cells (Donelan et al. 2002).

Very recently, a beta-cell specific knockout of the mouse homologue of kinesin heavy chain, Kif5b, was reported to cause glucose intolerance. In this *in vivo* study, the authors reported impairment of both early and sustained phases of insulin secretion although the sub-cellular localization of insulin granules was not affected by the decreased kinesin activity (Cui et al. 2011). It is thus possible that kinesin down-regulation affects insulin secretion because of defects other than insulin granule transport.

6-Beta-cell microfilaments and insulin secretion

The involvement of beta-cell microfilaments in insulin secretion was first suggested on the basis of experiments using drugs that alter actin polymerization. More recently, much attention has been paid to actin-dependent motor proteins as well as several actin-related regulatory factors hypothesized to contribute to the final steps of stimulus-secretion coupling.

6.1-Effect of microfilament-interfering drugs on insulin secretion

During the 1970's, several studies used microfilament-destabilizing cytochalasin B to test the possible implication of microfilaments in insulin granule movement towards the exocytotic sites on the plasma membrane. Cytochalasin B was

found to increase both phases of glucose-stimulated insulin secretion from isolated rat islets and the perfused rat pancreas (Malaisse et al. 1972, Lacy et al. 1973, Van Obberghen 1975) and to enhance tolbutamide (Lacy et al. 1973, Van Obberghen et al. 1973) and leucine (Van Obberghen et al. 1975) induced secretion.

Control studies then identified side-effects of the drug. Cytochalasin B (30 µM) was reported to inhibit initial glucose uptake in rat islets without affecting glucose oxidation of the sugar (McDaniel 1974). Others found that cytochalasin B impaired glucose metabolism by inhibiting its uptake, utilization and oxidation and partially inhibited Ca^{2+} uptake in rat islets but increased insulin secretion (Levy et al. 1976) with no detectable effect on granule saltatory movements (Somers et al. 1979). In my own studies, I did not detect effects of 10 µM cytochalasin on glucose metabolism in mouse islets as assessed by NAD(P)H measurements (Mourad et al. 2010). In fact, metabolic effects of cytochalasin B occur in non-beta cells of the islets (Jijakli et al. 2002).

Discrepant data were obtained in a study comparing the effect of microfilament destabilization in rat islets and poorly granulated HIT-T15 cells: insulin secretion was significantly inhibited by cytochalasins and C2 toxin in the cell line as opposed to the increase in secretion produced by these same drugs in primary islets. The conclusion was that microfilaments are required to recruit insulin granules in poorly granulated HIT-T15 cells, whereas they act as a barrier in primary beta-cells (Li et al. 1994).

Several recent studies dissipated any residual doubts. The more specific microfilament disrupter, latrunculin was consistently found to increase glucose and KCl-induced insulin secretion in clonal beta-cell lines as well as in rat, mouse and human islets (Lawrence et al. 2003, Thurmond et al. 2003, Tomas et al. 2006, Jewell et al. 2008). This increase produced by latrunculin was observed during both phases of glucose-induced secretion and was accompanied by disappearance of the cortical actin network and granule redistribution to the cell periphery (Jewell et al. 2008). The observation that latrunculin also potentiated secretion induced by a fixed Ca^{2+} concentration in permeabilized MIN6 cells suggested that the effect of microfilament

disrupters does not involve $[Ca^{2+}]_c$ changes but affects distal steps of the secretory response (Thurmond et al. 2003). At the single beta-cell level, Ca^{2+}-evoked exocytosis, measured by membrane capacitance recordings, was increased after treatment with cytochalasin D or latrunculin B (Ivarsson et al. 2005a). These observations corroborated the concept of an inhibitory cortical actin barrier.

An additional effect might contribute to the increase in insulin secretion by latrunculin. The drug was recently found to cause a redistribution of PIP2 to cover a larger area of the beta cell membrane, which might augment available docking sites for granules (Lopez et al. 2010). However, this view assumes that granules must be docked prior to exocytosis, a concept that is being revised (Izumi 2011, Seino et al. 2011).

The use of microfilament stabilizers has been less extensive mainly because of the rarity of adequate substances. Phalloidin, that is often used to stain filamentous actin (F-actin), stabilizes microfilaments but does penetrate intact cells. The drug was however shown to increase Ca^{2+}-evoked secretion from permeabilized rat islets (Stutchfield and Howell 1984). More recently, the use of jasplakinolide, a cell-permeant F-actin stabilizing agent, yielded discrepant and somehow unexpected results. KCl-induced insulin secretion was inhibited by jasplakinolide in RINm5F and MIN6 cells (Wilson et al. 2001, Lawrence and Birnbaum 2003, Nevins and Thurmond 2003), whereas glucose-induced secretion was increased by the microfilament stabilizer in MIN6 cells and mouse islets (Nevins and Thurmond 2003). Disappointingly, these observations that do not fit well the concept of disassembly of the cell web by glucose are often ignored in models of stimulus-secretion coupling in beta-cells (Wang and Thurmond 2009).

6.2-Actin dynamics upon glucose stimulation

Early studies using a DNAase-inhibition assay to determine the proportion of globular (G) and filamentous (F) actin in pancreatic islets reported a shift toward F-actin formation upon glucose stimulation (Howell and Tyhurst 1980, Swanston-Flatt et al. 1980). These results, together with the observation of an ATP-dependent

binding of insulin granules to actin filaments (Howell and Tyhurst 1979), were compatible with the proposed role of microfilaments in insulin granule exocytosis.

In complete disagreement with these early results, recent confocal microscopy studies revealed that glucose transiently but markedly diminishes cortical F-actin (Nevins and Thurmond 2003, Tomas et al. 2006), an effect mimicked by latrunculin (Thurmond et al. 2003, Tomas et al. 2006) but not elicited by KCl or non-metabolizable analogs of glucose (Wang and Thurmond 2009). Since glucose had no detectable effect on the F-actin:G-actin ratio (Nevins and Thurmond 2003), it has been proposed that subtle actin remodeling rather than global depolymerization of microfilaments is involved in insulin secretion (Wang and Thurmond 2009). Such a remodeling would allow insulin granules residing in regions deeper than the F-actin barrier to access the plasma membrane where they would eventually replenish the RRP and sustain insulin secretion particularly during the second phase (Seino et al. 2011).

Among the actin-related regulatory proteins mentioned before, gelsolin has been linked to exocytosis in beta-cells. MIN6 cells lacking this Ca^{2+}-dependent actin remodeling protein display increased cortical microfilaments that fail to depolymerize upon glucose stimulation. These cells also fail to secrete insulin in response to glucose unless their cortical actin network has been disrupted by latrunculin prior to glucose stimulation (Tomas et al. 2006). Further studies are needed to determine whether gelsolin-mediated actin remodeling affects one or both phases of insulin secretion.

Rho, Rac1 and Cdc42, all members of the small Rho-family GTPases, have also been implicated in beta-cell actin remodeling. These GTPases cycle between the GDP-bound (inactive) and GTP-bound (active) states and are known to translocate to the plasma membrane upon their activation (Kowluru 2010).

Glucose was shown to quickly activate Cdc42 in MIN6 cells then to glucosylate the GTPase on Thr37 (Just et al. 1995) which then passes to the GDP-bound form and becomes inactivated 5 minutes after stimulation (Nevins and Thurmond 2003). Furthermore, insulin secretion was inhibited when a constitutively

active form of Cdc42 was expressed in MIN6 cells, meaning that glucose might inactivate Cdc42 and thereby decrease actin polymerization to induce insulin secretion (Nevins and Thurmond 2003). Later on, a study from the same group presented evidence that Cdc42 is specifically required for the second phase of insulin secretion and that activation of this G-protein by glucose initiates a cascade involving activation of the downstream kinase, Pak1, after 5 minutes, followed by Rac1 15 minutes later when the second phase had already begun (Wang et al. 2007).

Rho and its associated kinase ROCK are also involved in cytoskeleton remodeling (Kowluru 2010). Their pharmacological inhibition increases both phases of glucose-induced insulin secretion and causes actin depolymerization in primary beta-cells (Hammar et al. 2009).

Adding to the complexity of the issue, it is relevant to mention the recent identification of Rho partners, the ERM (ezrin, radixin and moesin) proteins in beta-cells, (Lopez et al. 2010). In response to glucose stimulation, ERM proteins are phosphorylated in a Ca^{2+}-dependent manner and translocate to the cell periphery. Overexpression of dominant negative ezrin leads to a reduction in insulin granules near the membrane and impaired secretion. The involvement of ERM proteins in the secretory response could be through the link they create between cortical F-actin and PIP2 in the plasma membrane, the latter being a preferential docking site for secretory granules (Lopez et al. 2010).

Other molecular actors of exocytosis could benefit from glucose-induced actin remodeling. Glucose transiently decreases the colocalization of the t-SNARE proteins syntaxin1 and SNAP-25 as well as syntaxin4 with polymerized actin in MIN6 cells and primary islets (Thurmond et al. 2003, Jewell et al. 2008). Disruption of F-actin-syntaxin4 binding by glucose is suggested to free syntaxin and allows it to mediate granule fusion to the plasma membrane at a higher rate (Jewell et al. 2008).

6.3-Involvement of actin-related motor proteins.

The role of actin microfilaments is probably not limited to that of a regulatory physical barrier. They also permit insulin granule transport across the submembrane

region. Myosins, actin-regulated ATPases, are believed to be involved in vesicle transport (Rutter 2004).

In primary and clonal beta-cells, insulin secretagogues induce the phosphorylation of myosin II heavy chain (Wilson et al. 1999a) that colocalizes with F-actin (Wilson et al. 2001). Class V myosin motors have also been proposed as candidates for granule transport along microfilaments. Expression of a dominant-negative form of myosin Va or its inactivation by RNA silencing decreases the number of granules beneath the plasma membrane and causes a significant decrease in glucose-stimulated insulin secretion and depolarization-evoked exocytotic events in MIN6 cells (Varadi et al. 2005). Similar results were obtained by another group using INS-1 cells (Ivarsson et al. 2005a) in which inhibition of myosin Va interaction with insulin granules, by means of RNAi downregulation of myosin Va, reduced the number of granules recruited for release and single-cell exocytosis. These findings argue in favor of a dual role of the actin network in insulin secretion: a physical barrier that limits granule interaction with the plasma membrane and a track for insulin granule transport to the release sites.

VI. PERSONAL RESULTS

The aim of my thesis work was to better understand the mechanisms underlying the metabolic amplification of insulin secretion and to assess the contribution of this pathway to the two phases of secretion. The results can be subdivided in several parts, the first two of which have been published (Mourad et al. 2010; 2011). The other parts still need to be completed before their submission for publication.

The first study assessed the involvement of the amplifying pathway in both phases of glucose-induced insulin secretion. It also tested the hypothesis that metabolic amplification accelerates granule recruitment via effects on beta-cell actin microfilaments (Mourad et al. 2010)*.

The second study assessed the contribution of microtubules to both phases of insulin secretion and investigated their implication in amplification by glucose (Mourad et al. 2011)*.

Unpublished results presented in this thesis include a study of the role of AMPK in the amplification of insulin secretion by glucose. Two other studies deal with the role of microfilaments in the amplification of insulin secretion via the cAMP and PKC pathway respectively. In these last two studies, I also re-evaluated the involvement of cAMP and PKC in metabolic amplification.

I also coauthored an article reporting studies on the role of beta-cell microfilaments in insulin secretion triggered by intracellular Ca^{2+} mobilization or store-operated Ca^{2+} entry (Henquin et al. 2012). These results have not been included in the thesis for the sake of homogeneity.

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* The results published in these two articles are presented in a slightly modified, more concise way in the following pages, and eventually interpreted together in a global discussion. A reprint of each paper with a description of methods used can be found at the end of the thesis.

Study 1

Metabolic amplification and beta-cell actin microfilaments

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by NI Mourad, M Nenquin and JC Henquin

The aim of my first study was to investigate whether the metabolic amplifying pathway requires functional actin filaments in beta-cells. As explained in chapter V, most actin microfilaments in beta-cells are organized in a dense web beneath the plasma membrane (Orci et al. 1972). Pioneer studies by Lacy, Malaisse and Orci showed that cytochalasins, which depolymerize filamentous actin, facilitate insulin secretion and prompted the suggestion that this filamentous web actually limits the access of granules to exocytotic sites (Lacy et al. 1973, VanObberghen et al. 1973). Extending these early models (Malaisse et al. 1974, VanObberghen et al. 1975), more recent work using single beta-cells or insulin-secreting cell lines have proposed that granule translocation along actin filaments or remodeling of the web of actin filaments is involved in the replenishment of releasable granular pools, raising the possibility that these events contribute to the amplifying action of glucose (Eliasson et al. 2008, Varadi et al. 2002, Wang and Thurmond 2009).

That hypothesis was tested in the present study by treating mouse islets with latrunculin B and cytochalasin B to depolymerize actin, or with jasplakinolide to polymerize actin. The islets were then used to study the effects of glucose, tolbutamide and KCl during first and second phases of insulin secretion. $[Ca^{2+}]_c$ was measured in parallel experiments to ascertain that the observed changes in secretion were really due to the amplifying pathway and not to changes in the triggering Ca^{2+} signal.

1.1-RESULTS

1.1.1-Effects of drug treatment on islet actin

Mouse islets were preincubated for 90 min in the presence of actin-polymerizing or -depolymerizing drugs. We first ascertained their effects on the polymerization state of islet actin. In control islets, 24 ± 1.4 % of actin was present in filamentous form (Fig. 10). This proportion was decreased to 6 ± 1.6 and 10 ± 2.3 % after treatment with latrunculin B and cytochalasin B, respectively. In contrast, 95 ± 2.0 % of islet actin was in filamentous form after treatment with jasplakinolide, and this effect was not reversed (92 ± 2.9 %) by 60 min of washing without the drug (Fig. 10). The differences between these proportions are significant for all test groups ($p < 0.001$, Fisher's exact test).

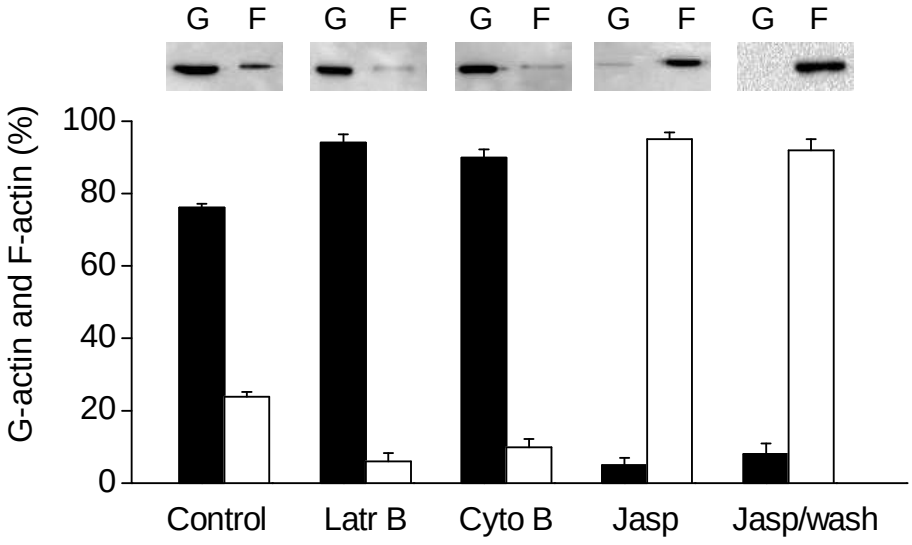

Fig. 10: Proportion of polymerized (filamentous - F) to depolymerized (globular - G) actin in control mouse islets and in islets treated with 2 µM latrunculin B (Latr B), 10 µM cytochalasin B (Cyto B) and 1 µM jasplakinolide (Jasp). Islets were incubated for 90 min in control medium containing 10 mM glucose and supplemented or not with the drugs, before being lysed and submitted to ultracentrifugation to separate the two actin fractions. Extracts were then subjected to western blotting for actin. Bars show the percentages of G and F actin in 3-5 experiments, of which one representative blot is shown at the top of the figure. In the group Jasp/wash, the islets were treated with jasplakinolide and then washed without drug for 60 min before lysis. The representative blot was not obtained in the same experiment as the other four. Values are means ± SE.

1.1.2-Impact of actin depolymerization on the metabolic amplification of insulin secretion during elevation of islet $[Ca^{2+}]_c$ by KCl

In this first series, K_{ATP} channels were held open with diazoxide and islet cells were depolarized with KCl in the presence of low or high glucose (Fig. 11). The resulting increase in $[Ca^{2+}]_c$ is shown in the inset of Figure 11A. It was similar in the presence of 1 and 15 mM glucose. However, insulin secretion was larger in the presence of high glucose (Fig. 11A). The difference, which corresponds to metabolic amplification, was significant and averaged 2.0 x for the whole period of KCl-induced insulin secretion (Fig. 11D).

Similar experiments were then performed after depolymerization of actin microfilaments with latrunculin B or cytochalasin B, which were present during the preincubation period and during $[Ca^{2+}]_c$ and insulin measurements. Latrunculin B (2 µM) augmented KCl-induced insulin secretion in low and high glucose (compare Fig. 11A and 11B) without affecting the increase in $[Ca^{2+}]_c$ (Fig. 11C). Notably, the amplifying action of glucose (ratio of the responses in low and high glucose) was increased by latrunculin B, as compared with controls (Fig 11D).

Treatment of the islets with cytochalasin B produced similar results. KCl-induced $[Ca^{2+}]_c$ rise was unaffected (Fig. 11C) but KCl-induced insulin secretion was augmented, and more so in high than low glucose (Fig. 11D), so that the amplifying action of glucose was larger than in control islets. This first series of experiments suggests that depolymerization of actin microfilaments might facilitate the metabolic amplification of insulin secretion.

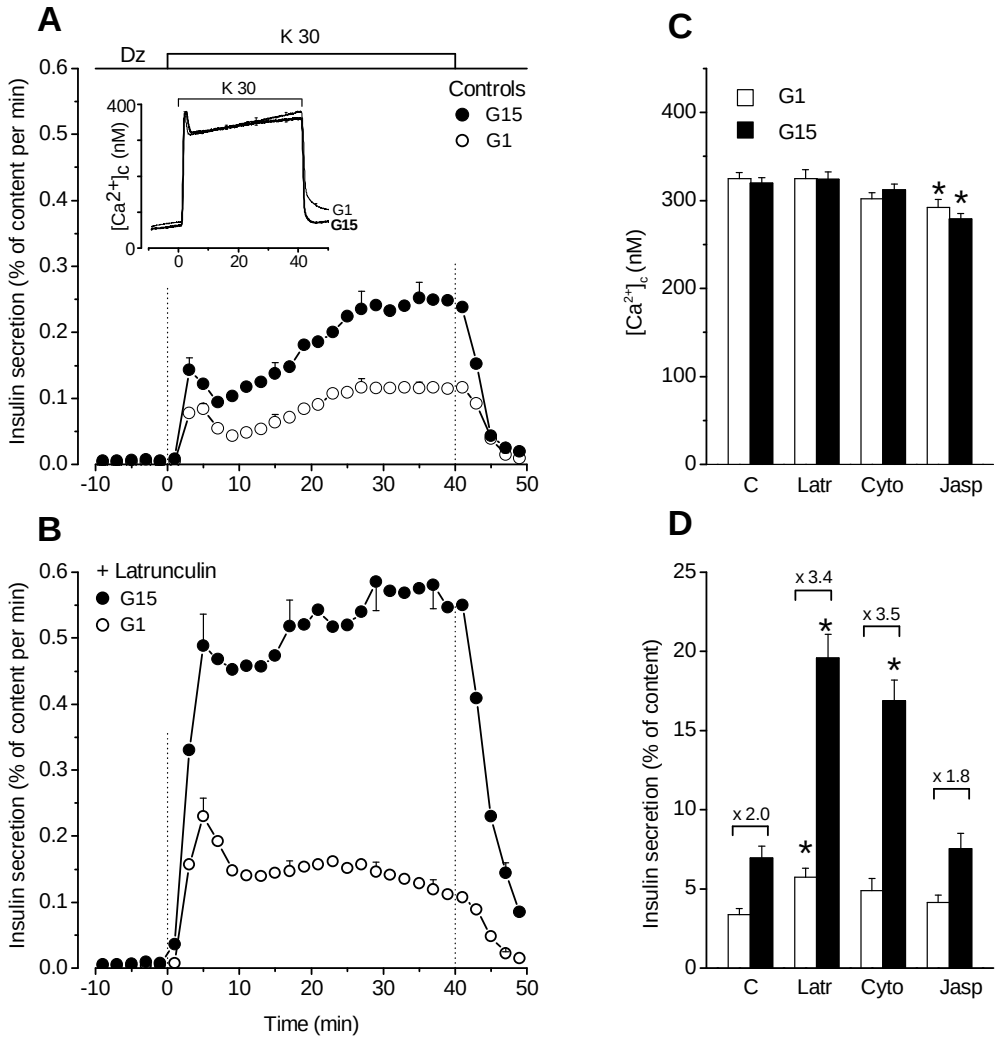

Fig. 11: Effects of actin depolymerization and polymerization on glucose amplification of insulin secretion during islet $[Ca^{2+}]_c$ clamping by KCl. Islets were first preincubated for 90 min with or without 2 µM latrunculin B (Latr), 10 µM cytochalasin B (Cyto) or 1 µM jasplakinolide (Jasp). They were then used to measure insulin secretion (A, B and D) or $[Ca^{2+}]_c$ (C and inset in A). The experiments were performed in the presence of 100 µM diazoxide (Dz) to prevent any effect of glucose on K_{ATP} channels, and either 1 or 15 mM glucose (G1 or G15). Islets were stimulated by a rise of the KCl concentration from 4.8 to 30 mM (K30) at 0 min. Latrunculin B and cytochalasin B were present during the experiments but jasplakinolide was present during the preincubation period only. Panels A (and inset) and B show the time course of insulin and $[Ca^{2+}]_c$ changes induced by KCl. Panels C and D show $[Ca^{2+}]_c$ and insulin responses integrated over the 40 min of stimulation. * above a column indicates a significant difference ($P < 0.05$ or less) with controls. Significant differences between responses in G1 and G15 are shown above pairs of columns, together with the fold-difference in insulin secretion (D).Values are means ± SE for 9 experiments of insulin secretion and 30-40 islets from 5-7 preparations for $[Ca^{2+}]_c$.

1.1.3-Impact of actin depolymerization on the metabolic amplification of insulin secretion during elevation of islet $[Ca^{2+}]_c$ by tolbutamide

In the second series, all beta-cell K_{ATP} channels were closed by addition of a high concentration (500µM) of tolbutamide to a medium containing 3mM glucose. The experiments illustrated in Fig. 12 (A and B) start 10 min after addition of 500µM tolbutamide to a medium containing 3mM glucose. In control islets, $[Ca^{2+}]_c$ was markedly elevated and insulin secretion was stimulated (compare with dotted lines labeled G3 and showing control data in the absence of tolbutamide). Subsequently raising the concentration of glucose to 15mM caused a rapid drop in $[Ca^{2+}]_c$ that reflects Ca^{2+} uptake by the endoplasmic reticulum and transient beta-cell membrane repolarization (Dufer et al. 2009b, Dukes et al. 1997). This drop was transient and followed by a return to levels slightly higher than before glucose stimulation (Sato et al. 1999) (Fig. 12A). Simultaneously with the $[Ca^{2+}]_c$ re-ascension, insulin secretion increased well above initial levels (Fig. 12B). The difference in secretory rate between steady-state periods in tolbutamide + high glucose (10-30 min) and tolbutamide + low glucose (-20-0 min) averaged 3.9-fold (Fig. 12D) for only a minor, though significant, increase in $[Ca^{2+}]_c$ (Fig. 12C). It is thus largely attributable to metabolic amplification (Sato et al. 1999).

Cytochalasin B treatment did not affect the rise in $[Ca^{2+}]_c$ (Fig. 12A) but strongly potentiated insulin secretion (Fig. 12B) induced by tolbutamide in 3mM glucose. When the concentration of glucose was then raised to 15mM, $[Ca^{2+}]_c$ and insulin secretion transiently decreased before increasing to prestimulatory levels for $[Ca^{2+}]_c$ (Fig. 12A and C) and much higher for the insulin secretion rate (Fig. 12B and D). Similar results were obtained in islets treated with latrunculin B. As a result, the amplifying effect of glucose after actin depolymerization, was of a similar relative magnitude (3.2 and 4.1-fold) as in control islets (3.9-fold) (Fig. 12D). Note, however, that these similar relative changes correspond to larger absolute amounts of insulin (Fig. 12B).

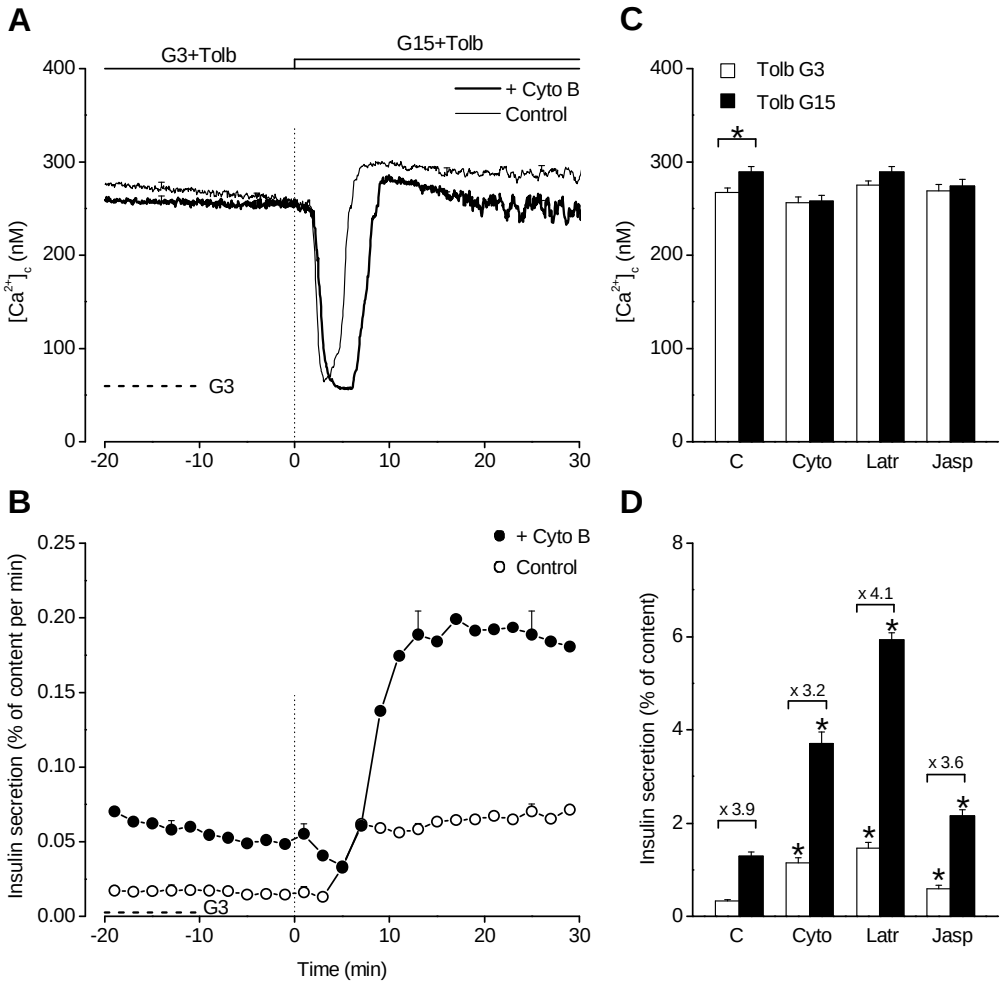

Fig. 12: Effects of actin depolymerization and polymerization on glucose amplification of insulin secretion during islet $[Ca^{2+}]_c$ elevation by tolbutamide. Islets were first preincubated for 90 min with or without 2 µM latrunculin B (Latr), 10 µM cytochalasin B (Cyto) or 1 µM jasplakinolide (Jasp). They were then used to measure $[Ca^{2+}]_c$ (A and C) or insulin secretion (B and D). Islets were stimulated with 500 µM tolbutamide (Tolb) in the presence of 3 mM glucose (G3) from -30 min, but only the last 20 min of this period are shown. The concentration of glucose was then raised to 15 mM (G15) at 0 min. Latrunculin B and cytochalasin B were present during the whole experiments, whereas jasplakinolide was present during the preincubation period only. Panels A and B show the time course of $[Ca^{2+}]_c$ and insulin secretion changes in control islets and in islets treated with cytochalasin B. Panels C and D show $[Ca^{2+}]_c$ and insulin responses integrated over the last 20 min in G3 + Tolb (-20-0 min) and in G15 + Tolb (10-30 min). * above a column indicates a significant difference ($P<0.01$) with controls. Significant differences ($P<0.01$) between values in G3 and G15 are shown above pairs of columns, together with the fold-change in insulin secretion induced by high glucose (D). Values are means ± SE for 8-10 experiments of insulin secretion and 30-38 islets from 5-7 preparations for $[Ca^{2+}]_c$.

1.1.4-Impact of actin depolymerization on the metabolic amplification of insulin secretion studied without clamping of islet $[Ca^{2+}]_c$

The amplifying action of glucose was also studied by comparing the changes in $[Ca^{2+}]_c$ and insulin secretion produced by separate stimulation of islets with either glucose or tolbutamide. As shown in Fig. 13A, the increase in $[Ca^{2+}]_c$ produced by 15mM glucose was delayed compared to that produced by 500µM tolbutamide in 3mM glucose, and its magnitude was slightly smaller during both first and second phases (Fig. 13B). The onset of insulin secretion was also slower after stimulation with high glucose than tolbutamide (Fig. 13C), but the magnitude of the response was larger (about 2-fold), during both first and second phases (Fig. 13D).

Islet pretreatment with latrunculin B or cytochalasin B did not significantly affect the tolbutamide-induced rise in $[Ca^{2+}]_c$ during either phase (Fig. 13B), but increased tolbutamide-induced insulin secretion 3 to 4-fold during both phases (Fig. 13D). Both agents also strongly augmented glucose-induced insulin secretion, and more so during the second phase (about 6-fold) than the first phase (about 3-fold) (Fig. 13D). This increase in secretion occurred although the glucose-induced rise in $[Ca^{2+}]_c$ was unaffected or slightly attenuated (Fig. 13B).

Comparison of the responses of control islets to the two stimuli shows that glucose induced larger first and second phases of insulin secretion than tolbutamide in the face of a smaller rise in $[Ca^{2+}]_c$ in control islets. Therefore, the amplifying action of glucose is a rapid and sustained phenomenon. The greater effect of glucose on insulin secretion in spite of a smaller increase in $[Ca^{2+}]_c$ persisted in test islets when the secretion rate had been augmented several-fold by depolymerization of actin microfilaments (Fig. 13D). As compared with controls, the difference was even larger during the second phase.

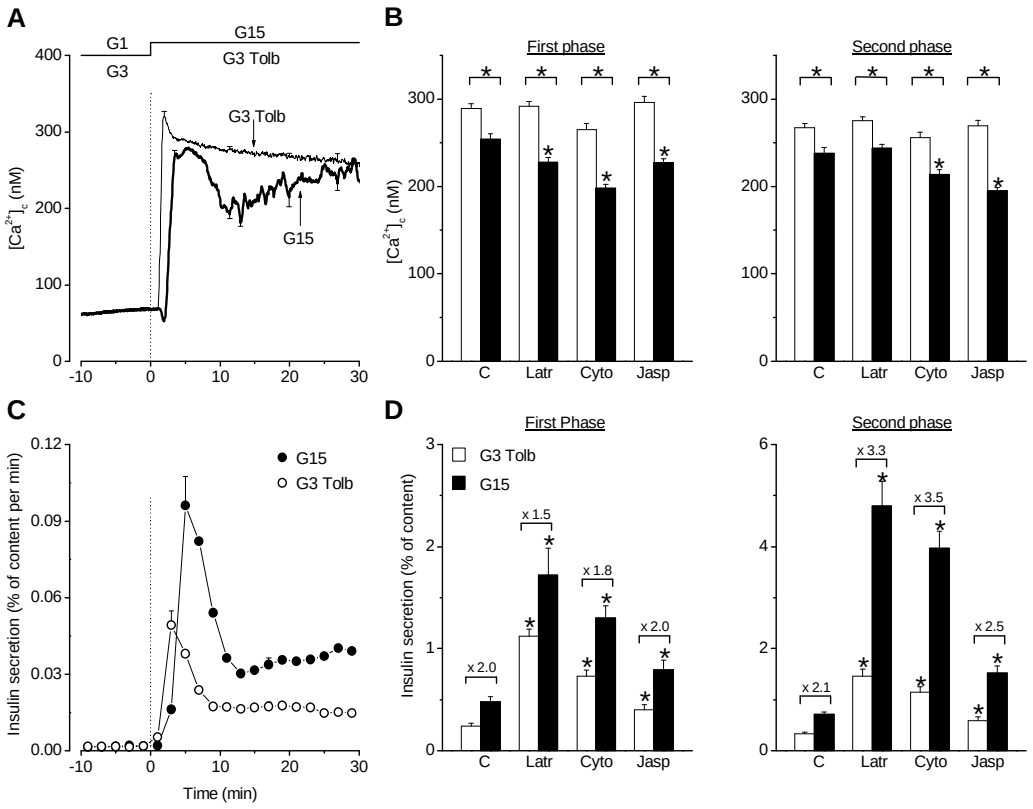

Fig. 13: Comparison of the effects of actin depolymerization or polymerization on glucose- and tolbutamide-induced insulin secretion. Islets were first preincubated for 90 min with or without 2 µM latrunculin B (Latr B), 10 µM cytochalasin B (Cyto B) or 1 µM jasplakinolide (Jasp). They were then used to measure $[Ca^{2+}]_c$ (A and B) or insulin secretion (C and D). Islets were stimulated with 500 µM tolbutamide (Tolb, thin line or open circles) in the presence of 3 mM glucose (G3), or by an increase in the glucose concentration from 1 to 15 mM (G1 – G15, thick line or filled circles). Latrunculin B and cytochalasin B were present during the whole experiments, whereas jasplakinolide was present during the preincubation period only. The experiments with tolbutamide are the same as those of Fig 3. Panels A and C show the time course of $[Ca^{2+}]_c$ and insulin changes in control islets. Panels B and D show $[Ca^{2+}]_c$ and insulin responses integrated over 7 min for first phase (2-9 min for tobutamide stimulation and 3-10 min for glucose stimulation) and 20 min for second phase (10-30 min for both). * above a column indicates a significant difference ($P < 0.01$) with controls. Significant differences ($P<0.01$) between responses to Tolb (open columns) and G15 (filled columns) are shown above pairs of columns, together with the fold-difference in insulin secretion (B and D).Values are means ± SE for 8-10 experiments of insulin secretion and 30-38 islets from 5-7 preparations for $[Ca^{2+}]_c$.

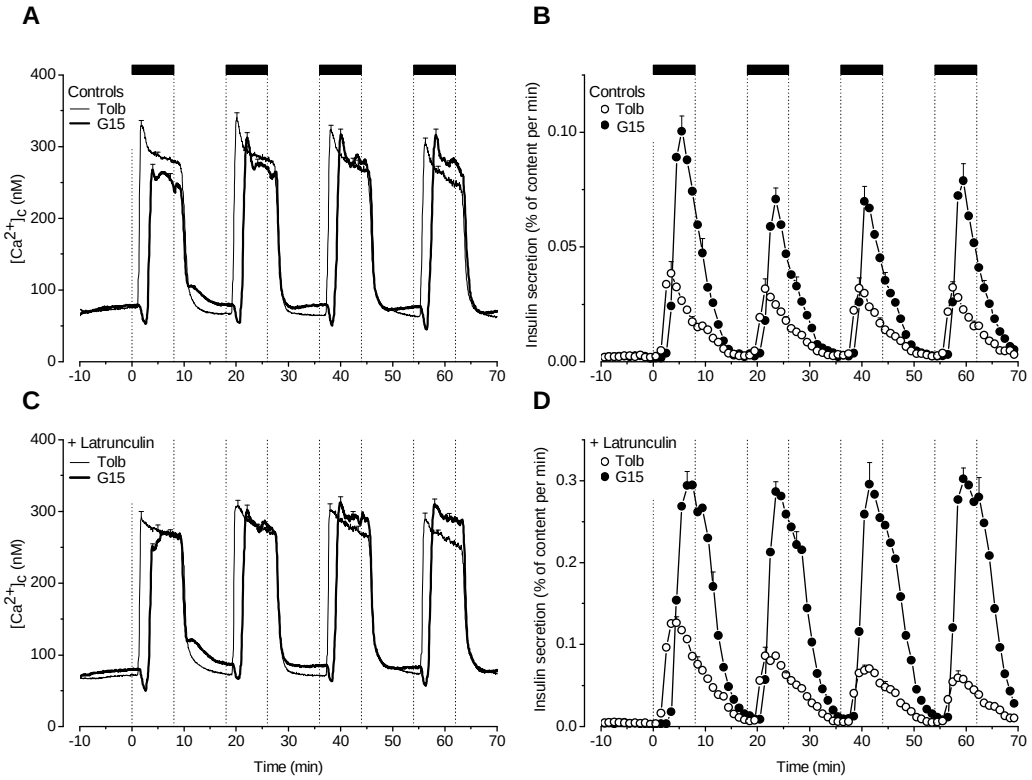

Fig. 14: Amplifying action of glucose during repetitive triggering of first phase of insulin secretion. Islet $[Ca^{2+}]_c$ (A and C) and insulin secretion (B and D) were measured in control islets (A and B) and in islets preincubated (90 min) and perifused with 1µM latrunculin B (C and D). The islets were stimulated by addition of 500µM tolbutamide to 3mM glucose (G) or by increasing the glucose concentration from 1 to 15mM. The stimulation was applied 4 times during periods of 8 min (black boxes) separated by 10-min periods of rest. Values are means ± SE for 30-32 islets from 4 preparations for $[Ca^{2+}]_c$ and 5 experiments of insulin secretion.

I next investigated whether this amplifying action of glucose during the first phase of insulin secretion persists when several first phases are triggered consecutively. Control islets were thus stimulated with 15mM glucose or with 500µM tolbutamide in low glucose during 4 periods of 8 min separated by 10min periods of rest. Each stimulation by glucose induced an increase in $[Ca^{2+}]_c$ that was delayed and smaller (first one) or similar (last three) to those triggered by tolbutamide (Fig. 14A). In contrast, insulin secretion was consistently 3 to 4-fold larger with glucose than tolbutamide (Fig. 14B). Similar experiments were then performed with latrunculin B-treated islets. As compared with control islets, the increases in $[Ca^{2+}]_c$ were unaffected, whereas insulin secretion triggered by either

glucose or tolbutamide was similarly augmented (Fig. 14D; note the difference in scale with Fig. 14B). These results show that the amplifying action of glucose is manifest during at least four successive first phases, even after depolymerization of actin microfilaments.

1.1.5-Impact of actin polymerization on the metabolic amplification of insulin secretion

Polymerization of actin was achieved by islet treatment with 1µM jasplakinolide (Fig. 10). However, in contrast to latrunculin B and cytochalasin B, jasplakinolide was present only during the preincubation period because its addition to perifusion solutions was prohibitively expensive. Nonetheless, Figure 10 shows that actin polymerization by jasplakinolide was not reversible after 1h of incubation in absence of the drug.

Jasplakinolide slightly inhibited the rise in $[Ca^{2+}]_c$ evoked by 30 mM KCl in low or high glucose (Fig. 11C) but did not influence KCl-induced insulin secretion so that glucose amplification of KCl-induced insulin secretion was unchanged compared with controls (Fig. 11D).

Pretreatment with jasplakinolide had no effect on steady-state $[Ca^{2+}]_c$ in the presence of tolbutamide (Fig. 12C) but slightly increased tolbutamide-induced insulin secretion in low glucose. The amplification of secretion produced by high glucose under these conditions was unaffected (3.6-fold in test vs 3.9-fold in controls) (Fig. 12D).

Pretreatment with jasplakinolide slightly decreased the glucose-induced rise in $[Ca^{2+}]_c$ (Fig. 13B), but augmented the two phases of insulin secretion by 1.7 and 2.2-fold (Fig. 13D). The Ca^{2+} response to tolbutamide was unaffected by jasplakinolide, but insulin secretion was again increased during both the first and second phases by 1.7 and 1.8-fold respectively so that the difference between secretory responses to glucose and tolbutamide was unaltered (Fig. 13D). The increase in the two phases of glucose-induced insulin secretion produced by pretreatment of the islets with

jasplakinolide is illustrated in Figure 15 (left-hand part, until 30 min). Jasplakinolide pretreatment also similarly augmented insulin responses to glucose and tolbutamide during successive brief stimulations like those in Fig. 14, so that the difference between the effects of the two agents persisted during repetitive first phases (not shown). The amplifying action of glucose on the first phase can thus manifest itself when actin microfilaments are kept polymerized.

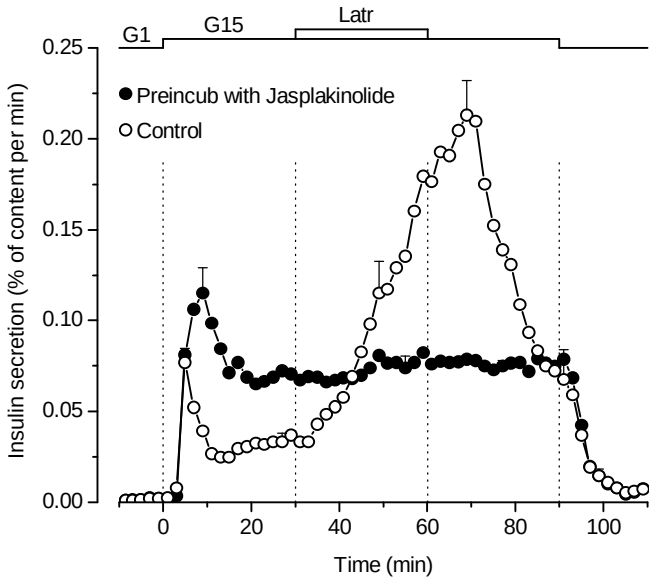

Fig. 15: Preincubation with jasplakinolide prevents the acute effects of latrunculin B on insulin secretion. One group of islets (filled circles) was preincubated for 90 min in the presence of 1 µM jasplakinolide, while the other group (open circles) was preincubated without drug before being used to study insulin secretion. Latrunculin B (2 µM) was added and the concentration of glucose changed as indicated. Values are means ± SE for 4 experiments.

The right-hand part of Figure 15 (after 30 min) shows that the acceleration of insulin secretion, which followed acute addition of latrunculin B to control islets, did not occur in islets preincubated with jasplakinolide. Although these and the above results establish that the effects of a pretreatment with jasplakinolide persist during perifusions subsequently performed in the absence of the drug, a final series of

experiments was carried out in its presence. Islets were preincubated with 1µM jasplakinolide (as in other experiments) and incubated (rather than perifused) also in its presence. As can be seen in Table 1 of the published paper (available at the end of the thesis) the results were similar to those obtained with islets preincubated with and perifused without jasplakinolide.

1.2-DISCUSSION

The results of my first study show that amplification of insulin secretion by glucose is a rapid and sustained phenomenon, which influences the magnitude of both first and second phases of the secretory response, without the requirement of functional actin microfilaments in beta-cells. They suggest that metabolic amplification is a late event in stimulus-secretion coupling, possibly corresponding to acceleration of the priming process that confers release competence to insulin granules.

1.2.1-Effects of test agents on actin polymerization

Early electron microscopic studies showed that cytochalasin B (20µM) disrupts the web of microfilaments underneath the plasma membrane in rat beta-cells (VanObberghen et al. 1973). More recent morphological and biochemical approaches have established that latrunculin B (10µM) decreases actin polymerization (Jewel et al. 2008, Tomas et al. 2006, Tsuboi et al. 2003) whereas jasplakinolide (5µM) increases actin polymerization (Nevins and Thurmond 2003) in MIN6 insulin-secreting cells. We now show that, in mouse islets, lower concentrations of cytochalasin B (10µM) and latrunculin B (2µM) decrease the proportion of polymerized actin to less than 10 %, whereas jasplakinolide (1µM) causes virtually complete polymerization of actin.

1.2.2-Influence of actin (de)polymerization on insulin secretion

Cytochalasin B has long been known to increase the two phases of insulin secretion induced by high glucose or by a sulfonylurea at non-stimulatory (1.7–5.5mM) glucose (Lacy et al. 1973, VanObberghen et al. 1973). More recently, latrunculin B was found to increase KCl-induced and glucose-induced insulin secretion in MIN6 cells (Lawrence and Birnbaum 2003, Thurmond et al. 2003, Tomas et al. 2006). In our study, both cytochalasin B and latrunculin B augmented the rapid first phase of insulin secretion induced by high glucose or tolbutamide. The sustained second phase was also augmented, except during stimulation with KCl in low glucose. There is thus agreement that drugs depolymerizing actin filaments in beta-cells augment insulin secretion.

In contrast, there is no consensus concerning the impact of actin polymerization by jasplakinolide. The drug was reported to inhibit KCl-induced insulin secretion in RIN cells (Wilson et al. 2001) and MIN6 cells (Lawrence and Birnbaum 2003) and to block glucose-induced vesicle release in MIN6 cells (Tsuboi et al. 2003). These inhibitory effects of jasplakinolide in cell lines contrast with the potentiation of glucose-induced insulin secretion observed in incubated mouse islets (Nevins and Thurmond 2003). We confirm the latter observation and further show that jasplakinolide augments both phases of the secretory response to glucose and tolbutamide, but does not significantly change the response to KCl. Further experiments performed after the publication of this paper have, however, shown that the increase in KCl-induced insulin secretion caused by jasplakinolide is statistically significant although smaller, in relative terms, than the increase caused by the drug in the case of glucose or tolbutamide stimulation. We have no explanation for this selective stimulation that cannot be attributed to distinct effects on $[Ca^{2+}]_c$.

It is thus clear that the peripheral network of actin microfilaments functions as a brake of insulin secretion in primary beta-cells (Lacy et al. 1973, Orci et al. 1972, VanObberghen et al. 1975), and our results show that this brake can be lifted by either polymerization or depolymerization of actin. Other actin microfilaments,

localized more deeply in the cytoplasm of beta-cells (Gabbiani et al. 1974), have been suggested to play a positive role in the translocation of insulin granules achieved by motor proteins such as kinesins or myosin 5A (Ivarsson et al. 2005a, Varadi et al. 2002, Varadi et al. 2005). However, these experiments have been performed with insulin-secreting cell lines that contain much fewer (<5%) insulin granules than primary beta-cells, and thus more heavily rely on physical translocation of granules to sustain high insulin secretion rates. This may explain differences in the responses of cell lines and islets to actin depolymerization (Li et al. 1994) or polymerization (Lawrence and Birnbaum 2003, Nevins and Thurmond 2003, Tsuboi et al. 2003, Wilson et al. 2001).

1.2.3-Influence of actin (de)polymerization on the triggering $[Ca^{2+}]_c$ signal

The possible impact of actin depolymerization on beta-cell $[Ca^{2+}]_c$ has been evaluated previously in only one study using HIT cells. Cytochalasin B did not impair the rise in $[Ca^{2+}]_c$ produced by mixed nutrients while paradoxically inhibiting insulin secretion (Li et al. 1994). The general idea that the stimulatory effects of the drug on insulin secretion are independent of changes in $[Ca^{2+}]_c$ therefore relies on other approaches. Cytochalasin B was found to increase insulin release induced by a fixed Ca^{2+} concentration in permeabilized rat islets (Yaseen et al. 1982), and latrunculin B to had similar effect in MIN6 cells (Thurmond et al. 2003). Similar observations were made when exocytosis was evaluated by capacitance recordings in dialysed INS-1 cells (Ivarsson et al. 2005). Our experiments in intact islets show that the increase in insulin secretion produced by actin-depolymerizing or -polymerizing drugs was never accompanied by an increase in $[Ca^{2+}]_c$, regardless of the stimulus, and sometimes even occurred in face of a small decrease in $[Ca^{2+}]_c$. We can thus safely conclude that actin remodeling affects insulin secretion by mechanisms distal to the rise in $[Ca^{2+}]_c$. Moreover, and most importantly for the present study, the small changes in beta-cell $[Ca^{2+}]_c$ that the drugs sometimes produced have no impact on our main question concerning the role of actin filaments in the amplifying action of

glucose. Indeed, this action of glucose on insulin secretion was never attributable to a further rise in $[Ca^{2+}]_c$.

1.2.4-Influence of actin (de)polymerization on metabolic amplification

We used three experimental paradigms to study amplification of insulin secretion by glucose, and never found it to be impaired by disruption of actin microfilaments. Persistence and even augmentation of amplification after islet treatment with latrunculin B or cytochalasin B do not support the hypothesis that amplification corresponds to glucose-mediated depolymerization of actin. The hypothesis also seems to be ruled out by the preservation of amplification after complete polymerization of actin. It is obvious that metabolic amplification does not require functional actin microfilaments to augment insulin secretion. If granule movements to the exocytotic sites take place during amplification, actin-independent mechanisms must be involved (Ivarsson et al. 2005, Varadi et al. 2005, Wang and Thurmond 2009). We do not exclude, however, that subtle remodeling of the actin network under the influence of glucose, rather than major shifts in the actin polymerization state, plays a role in stimulus-secretion coupling (Wang and Thurmond 2009).

Study 2

Metabolic amplification and beta-cell actin microtubules

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by NI Mourad, M Nenquin and JC Henquin

The aim of my second study was to investigate whether the metabolic amplifying pathway requires functional microtubules in beta-cells. The proposal that beta-cell microtubules are active players in insulin secretion originates from experiments by Lacy (Lacy et al. 1972) and Malaisse (Devis et al. 1974, Malaisse et al. 1971, Somers et al. 1974), who showed that drugs known to interfere with tubulin polymerization (vinblastine, vincristine and colchicine) generally inhibited insulin secretion from pieces of rat pancreas, isolated islets, or the perfused pancreas. Most of the results supported models in which the second phase of glucose-induced insulin secretion is dependent on the mobilization of secretory granules along microtubules (Howell and Tyhurst 1982, Malaisse et al. 1975). This interpretation was supported by cinematographic evidence that vinblastine and colchicine inhibited glucose-induced granular movements in beta-cells (Kanazawa et al. 1980, Lacy et al. 1975, Somers et al. 1979). More recently, conventional kinesin, that provides ATP-dependent motor activity to transport secretory vesicles along microtubules, has been identified in insulin-secreting cells lines and in islets (Balczon et al. 1992, Meng et al. 1997). It was then proposed that one consequence of the rise in beta-cell $[Ca^{2+}]_c$ produced by glucose is dephosphorylation of kinesin, with an increase in its motor activity and acceleration of insulin granule movements along microtubules (Donelan et al. 2002). This scenario was compatible with observations that inactivation of kinesin by antisense nucleotides or dominant-negative mutants inhibits insulin granule movement and insulin secretion (Meng et al. 1997, Varadi et al. 2002, Varadi et al. 2003). Glucose-induced granular movement along microtubules is thus a plausible mechanism of metabolic amplification.

To test that hypothesis, mouse islets were treated with nocodazole to depolymerize tubulin and disrupt microtubules, or with taxol to polymerize tubulin and stabilize microtubules (Jordan et al. 1998, Jordan and Kamath 2007). Insulin secretion and islet $[Ca^{2+}]_c$ were measured in dynamic perifusion systems to distinguish between the two phases of secretion and ascertain that the observed changes in secretion were really due to the amplifying pathway and not to alterations of the triggering Ca^{2+} signal.

2.1-RESULTS

2.1.1-Effects of drug treatment on islet tubulin

We first characterized the effects of the drugs we used on the polymerization state of islet tubulin. In control islets, 34 ± 2.7 % of tubulin was present in the form of microtubules (polymerized) and 66% was in free (unpolymerized) form (Fig.16). This proportion is similar to that previously found in rat islets (McDaniel et al. 1980, Pipeleers et al. 1976). After 90 min of preincubation with nocodazole, a microtubule disrupter, islet tubulin was almost completely depolymerized. Preincubation with taxol, a microtubule stabilizer, produced the opposite effect, causing virtually complete polymerization of islet tubulin (Fig.16, left). These biochemical results in islets agree with the morphologic disappearance and stabilization of microtubules in insulin-secreting HIT cells treated with nocodazole and taxol respectively (Farshori and Goode 1994). Figure 16 (right) also shows that latrunculin B and cytochalasin B, two drugs causing depolymerization of actin microfilaments (Mourad et al. 2010), did not influence tubulin polymerization, and did not interfere with the decrease and increase in tubulin polymerization produced by nocodazoleaandataxolarespectively. The differences between control and test groups are highly significant ($p < 0.001$ by Fisher's exact test).

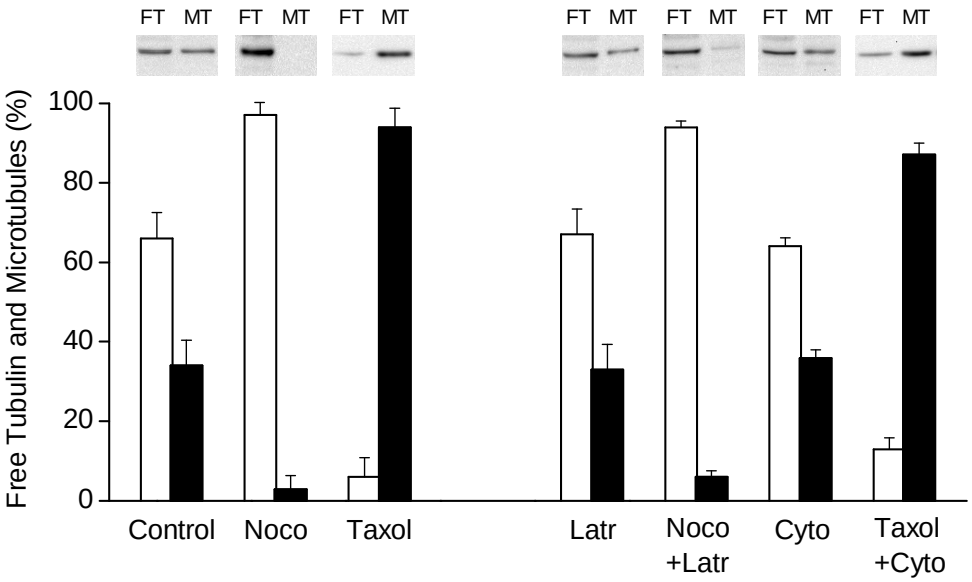

Fig. 16: Proportion of unpolymerized tubulin [free tubulin (FT)] to polymerized tubulin [microtubular (MT)] in mouse islets. Left: Control islets and islets treated with 10 µM nocodazole (Noco) or 5 µM taxol (Tax). Right: Islets treated with 1µM latrunculin B (Latr) or 10µM cytochalasin B (Cyto) alone or combined with nocodazole or taxol. Islets were incubated for 90 min in control medium containing 10 mM glucose and supplemented or not with the drugs. They were then lysed and submitted to ultracentrifugation to separate the two tubulin fractions. Extracts were later subjected to western blotting for tubulin. Bars show the percentages of FT and MT in 6 control and 3-5 test experiments, of which one representative blot is shown at top. Not all blots were obtained in the same experiment. Values are means ± SD.

2.1.2-Impact of microtubule disruption and stabilization on the metabolic amplification of insulin secretion during elevation of islet $[Ca^{2+}]_c$ by KCl

While K_{ATP} channels were held open with diazoxide, islet cells were depolarized with KCl in the presence of low or high glucose (Fig. 17). The resulting increase in $[Ca^{2+}]_c$ was similar in the presence of 1 and 15 mM glucose, but insulin secretion was larger in the presence of high glucose. The ~2-fold difference in secretion (Fig. 17D), for a similar $[Ca^{2+}]_c$ (Fig. 17C) corresponds to metabolic amplification.

Similar experiments were then performed after depolymerization of tubulin with 10µM nocodazole that was present during the preincubation period and during $[Ca^{2+}]_c$ and insulin measurements (Fig. 17A). Nocodazole slightly (15%, $P<0.05$)

attenuated the KCl-induced rise in $[Ca^{2+}]_c$ in 1mM glucose but not in 15mM glucose (Fig. 17C). Compared with controls, insulin secretion was not significantly affected, so that the amplifying action of high glucose (2.1-fold) was unaltered (Fig. 17A and 17D).

We then tested the effects of 5μM taxol, a microtubule-stabilizer (Fig. 17B). The time course of the KCl-induced $[Ca^{2+}]_c$ increase was unaffected (Fig. 17B, inset), but its magnitude was reduced by ~20% in low and high glucose (Fig. 17C). This may contribute to the ~50% inhibition of KCl-induced insulin secretion that taxol produced at both glucose concentrations (Fig. 17D). Importantly, the secretory response induced by a similar $[Ca^{2+}]_c$ (Fig. 17C) was doubled by high glucose as in controls (Fig. 17D). Metabolic amplification was thus unaltered by microtubule disruption or stabilization under these conditions of depolarization with KCl.

2.1.3-Impact of microtubule disruption and stabilization on the metabolic amplification of insulin secretion during elevation of islet $[Ca^{2+}]_c$ by tolbutamide

The experiments illustrated in Fig. 18 (A and B) start 10 min after the addition of 500μM tolbutamide to a medium containing 3mM glucose. In control islets, $[Ca^{2+}]_c$ was markedly elevated and insulin secretion was stimulated (compare to dotted lines labeled G3 and showing control data in the absence of tolbutamide). Subsequently raising the concentration of glucose to 15mM caused a rapid but transient drop in $[Ca^{2+}]_c$, which reflects Ca^{2+} uptake by the endoplasmic reticulum and transient beta-cell membrane repolarization (Dufer et al. 2009, Dukes et al. 1997). Concomitantly with the reascension of $[Ca^{2+}]_c$ to levels slightly higher than before glucose stimulation (Sato et al. 1999), insulin secretion increased markedly (Fig. 18B). The difference in $[Ca^{2+}]_c$ between steady-state periods in tolbutamide + high glucose (10-30 min) and tolbutamide + low glucose (-20-0 min) was small though significant (Fig. 18C), whereas the difference in secretory rate averaged 4.2-fold (Fig. 18D) and is thus largely attributable to amplification (Sato et al. 1999).

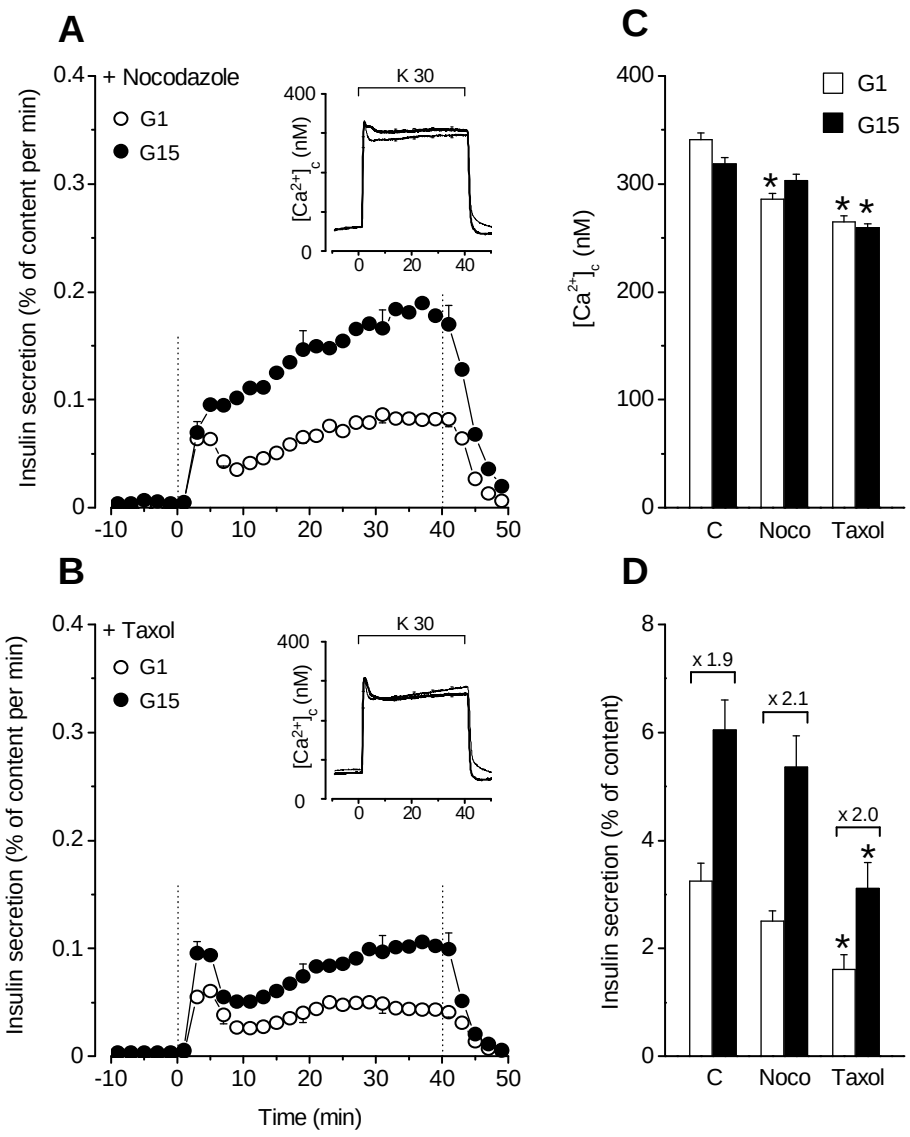

Fig. 17: Effects of tubulin depolymerization and polymerization on glucose amplification of insulin secretion during clamping of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in islets with KCl. Islets were first preincubated for 90 min with or without 10 µM nocodazole (Noco) or 5 µM taxol. They were then used to measure insulin secretion (A, B and D) or $[Ca^{2+}]_c$ (C and insets in A and B). Experiments were performed in the presence of 100 µM diazoxide (Dz) to prevent any effect of glucose on ATP-sensitive K^+ (K_{ATP}) channels, and either 1 or 15 mM glucose (G1 or G15). Islets were stimulated by a rise of the KCl concentration from 4.8 to 30 mM (K30) at 0 min. Nocodazole and taxol were present throughout the experiments. A and B (and insets): time course of insulin and $[Ca^{2+}]_c$ changes induced by KCl. C and D: $[Ca^{2+}]_c$ and insulin responses integrated over the 40 min of stimulation. * above a column indicates a significant difference ($P < 0.05$ or less) with controls. Significant differences ($P<0.001$) between responses in G1 and G15 are shown above pairs of columns, together with the fold-difference in insulin secretion (F). Values are means ± SE for 10 experiments of insulin secretion and 36-45 islets from 6-7 preparations for $[Ca^{2+}]_c$.

Islet treatment with nocodazole did not affect $[Ca^{2+}]_c$ or insulin secretion in the presence of tolbutamide and 3 or 15mM glucose (Fig. 18). The amplification of secretion (4.3-fold difference between responses in low and high glucose) was thus similar to that in control islets (Fig. 18D). Islet treatment with taxol slightly lowered $[Ca^{2+}]_c$ (Fig. 18A and C) and similarly inhibited (40 %) insulin secretion (Fig. 18B and D) in the presence of tolbutamide and low or high glucose. The amplifying action of glucose on secretion therefore remained unaltered (4.2-fold) (Fig. 18D).

2.1.4-Impact of microtubule disruption and stabilization on the metabolic amplification of insulin secretion studied without clamping of islet $[Ca^{2+}]_c$

The amplifying action of glucose was next studied by comparing the changes in $[Ca^{2+}]_c$ and insulin secretion produced by separate stimulation of islets with either 15mM glucose or 500µM tolbutamide in 3mM glucose (Fig. 19). In control islets, tolbutamide produced a rapid increase in $[Ca^{2+}]_c$ characterized by an initial peak followed by a steady but slowly declining elevation (Fig. 19A, trace C), and triggered biphasic insulin secretion (Fig. 19D, filled circles). Glucose also produced a biphasic increase in $[Ca^{2+}]_c$ that was slightly delayed, compared to the response to tolbutamide (Fig.19 B, trace C), because of a small initial decrease due to uptake of Ca^{2+} into the endoplasmic reticulum (Dukes et al. 1997, Gylfe 1988). In response to this rise in $[Ca^{2+}]_c$, biphasic insulin secretion occurred (Fig. 19E, filled circles), which was also delayed compared to the response to tolbutamide. Importantly, the effect of glucose on $[Ca^{2+}]_c$ was smaller than that of tolbutamide during both first and second phases (Fig. 19C, compare filled to open columns), whereas its effect on insulin secretion was more than 2-fold larger, also during both phases (Fig. 19F). This confirms that the amplifying action of glucose is a rapid and sustained phenomenon (Mourad et al. 2010).

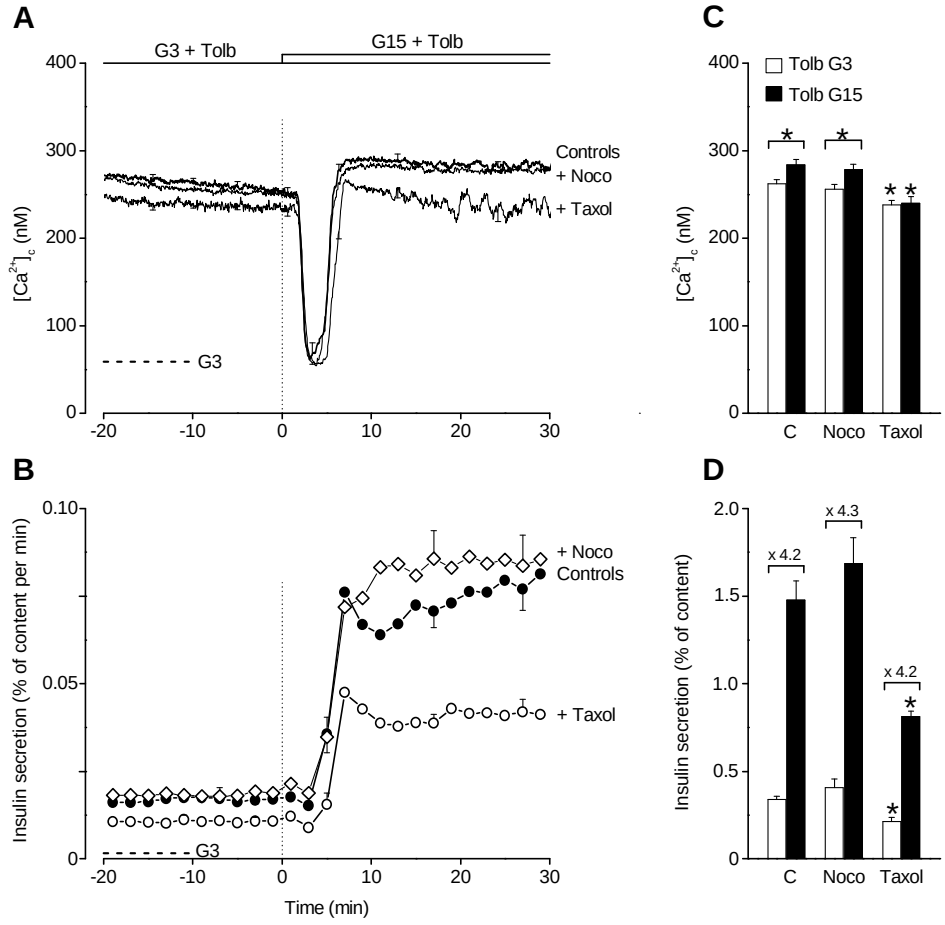

Fig. 18: Effects of tubulin depolymerization and polymerization on glucose amplification of insulin secretion during islet $[Ca^{2+}]_c$ elevation by tolbutamide. Islets were first preincubated for 90 min with or without 10 µM nocodazole (Noco) or 5 µM taxol. They were then used to measure $[Ca^{2+}]_c$ (A and C) or insulin secretion (B and D). Islets were stimulated with 500 µM tolbutamide (Tolb) in the presence of 3 mM glucose (G3) from -30 min, but only the last 20 min of this period are shown. The concentration of glucose was then raised to 15 mM (G15) at 0 min. Nocodazole and taxol were present throughout the experiments. A and B: time course of $[Ca^{2+}]_c$ and insulin secretion changes in control islets and islets treated with nocodazole or taxol. The dotted line labeled G3 shows $[Ca^{2+}]_c$ and insulin secretion in the absence of tolbutamide. C and D: $[Ca^{2+}]_c$ and insulin responses integrated over the last 20 min in G3 + Tolb (-20-0 min) and in G15 + Tolb (10-30 min). * above a column indicates a significant difference (P<0.01) with controls. Significant differences (P<0.01 or less) between values in G3 and G15 are shown above pairs of columns, together with the fold-change in insulin secretion induced by high glucose (D). Values are means ± SE for 8-10 experiments of insulin secretion and 30-40 islets from 5-7 preparations for $[Ca^{2+}]_c$.

Islet treatment with nocodazole affected neither the time-course nor the amplitude of tolbutamide- and glucose-induced $[Ca^{2+}]_c$ and insulin responses (Fig. 19), so that the amplifying action of glucose on insulin secretion remained unaltered (Fig. 19F). Islet treatment with taxol slightly attenuated the rise in $[Ca^{2+}]_c$ (Fig. 19A-C) and the insulin secretion response (Fig. 19 D-F) produced by the two secretagogues. The effect on $[Ca^{2+}]_c$ was smaller (~10%) than that on insulin secretion (~30%). However, in spite of this partial inhibition, the amplifying action of glucose on secretion was not altered (Fig. 19F). Altogether these results show that metabolic amplification does not depend on functional microtubules.

2.1.5-Impact of concomitant perturbation of microtubule and microfilament functions on the metabolic amplification of insulin secretion

In the first study, I showed that, while increasing insulin secretion, depolymerization of actin microfilaments did not impair metabolic amplification (Mourad et al. 2010). I now investigated the impact of a concomitant inactivation of microtubules and microfilaments (Fig. 20). Islet microfilaments were disrupted by treatment with either cytochalasin B or latrunculin B (Mourad et al. 2010). The lack of effect of these two drugs on tubulin polymerization has been described above (Fig. 16). We also ascertained that taxol and nocodazole had no effect on microfilaments. The proportion of polymerized to depolymerized actin (76%/24% in control islets) was unaffected by taxol (78%/22%) or nocodazole (75%/25%) alone. Neither drug impaired the depolymerizing action of cytochalasin B (96%/4%) or latrunculin B (99%/1%).

In a first series of experiments, we tested the impact of a concomitant interference of microtubules and microfilaments. Islets were preincubated and then perifused with 10µM cytochalasin B and 5µM taxol, while controls were preincubated and perifused without drugs or with only one of the two drugs (Fig. 20A-D). When tested alone, cytochalasin B increased tolbutamide- and glucose-induced insulin secretion ~ 4-fold (Fig. 20A-C), in spite of a slight inhibition of

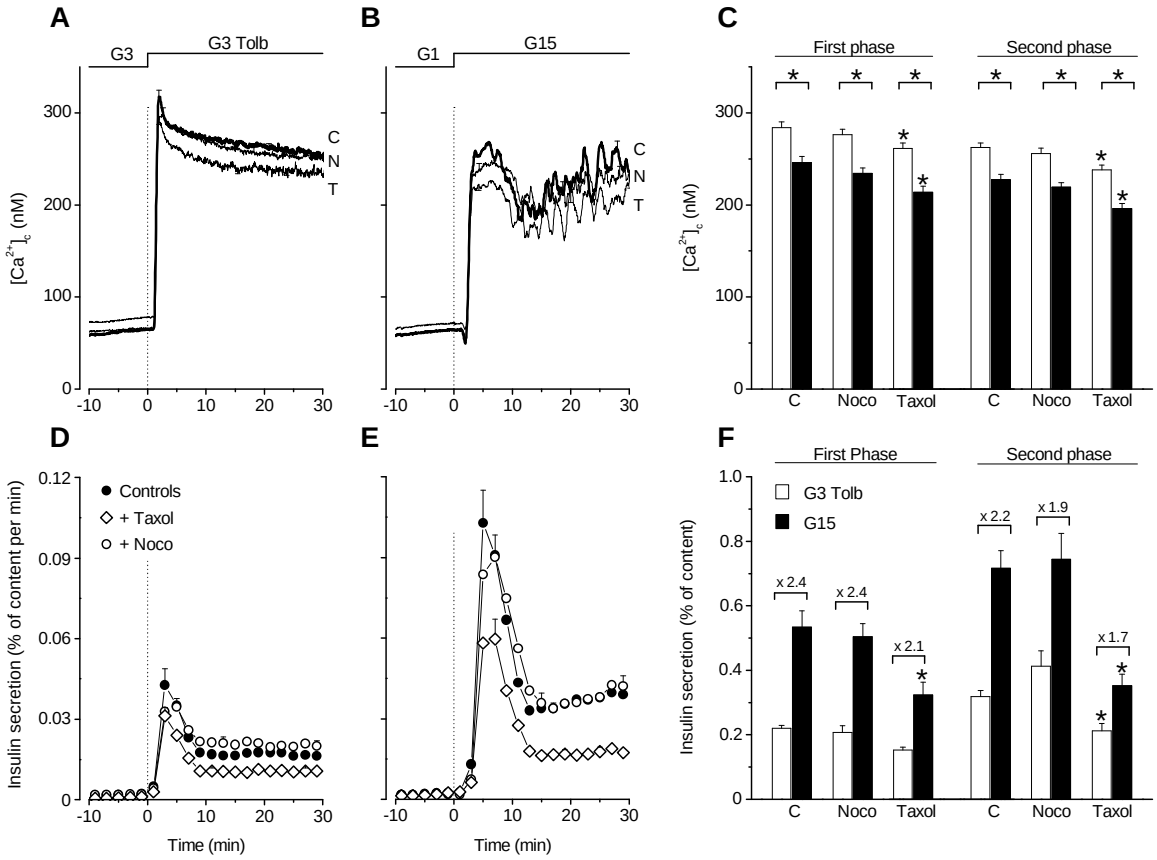

Fig. 19: Effects of tubulin depolymerization and polymerization on glucose- and tolbutamide-induced insulin secretion. Islets were first preincubated for 90 min with or without 10µM nocodazole (Noco) or 5µM taxol. They were then used to measure $[Ca^{2+}]_c$ (A, B and C) or insulin secretion (D, E and F). Nocodazole or taxol was present throughout the indicated experiments. A and D: islets were stimulated with 500µM tolbutamide (Tolb) in the presence of 3 mM glucose (G3). B and E: islets were stimulated by an increase in the glucose concentration from 1 to 15mM. A, B, D and E: time course of $[Ca^{2+}]_c$ and insulin changes. C and F: $[Ca^{2+}]_c$ and insulin responses integrated over 7 min for first phase (2-9 min for tobutamide stimulation and 3-10 min for glucose stimulation) and 20 min for second phase (10-30 min for both). * above a column indicates a significant difference ($P < 0.01$) with controls. Significant differences ($P<0.01$) between responses to Tolb in G3 (open columns) and to G15 (filled columns) are shown above pairs of columns, together with the fold-difference in insulin secretion (C and F).Values are means ± SE for 8-11 experiments of insulin secretion and 33-45 islets from 5-7 preparations for $[Ca^{2+}]_c$.

Note to panel F: The relative difference between the secretory responses to G15 and to G3 Tolb is not affected by taxol treatment when expressed as a fold-difference, but one should bear in mind that the drug decreases the absolute amount of insulin that is secreted (hence the number of granules).

$[Ca^{2+}]_c$ in the presence of glucose (Fig. 20D). Compared with untreated controls, the effect of cytochalasin B on insulin secretion induced by both secretagogues was slightly attenuated by taxol (increase by 3-fold only) (Fig. 20A-B), probably because of an inhibition of the $[Ca^{2+}]_c$ rise (Fig. 20D). However, when the combined effects of taxol + cytochalasin B were compared to the inhibited response of islets treated with taxol alone, the difference was 4-fold again.

In a second series of experiments, we interfered with microtubules *before* depolymerizing microfilaments. Islets were preincubated without drug or with 10µM nocodazole to disrupt microtubules, and then perifused with nocodazole, 1µM latrunculin B or both (Fig. 20E-H). In islets preincubated withoudrug, latrunculin B alone increased tolbutamide- and glucose-induced insulin secretion ~ 4-fold (Fig. 20E-G), without affecting $[Ca^{2+}]_c$ (Fig. 20H). Similar increases in insulin secretion by latrunculin B were observed in islets pretreated with nocodazole.

When islets were preincubated with taxol alone and then perifused with taxol and cytochalasin B, the results were similar to those shown in Fig 20A-C, when both drugs were present during the preincubation period (data not shown). We can thus conclude that microtubules are not required to sustain the high rates of insulin secretion occurring when the brake exerted by actin microfilaments is lifted.

As to the specific question addressed in the study, the results clearly establish that amplification of insulin secretion, estimated by the ratio of the responses to glucose versus tolbutamide, is neither augmented nor impaired by concomitant inactivation of microfilaments and microtubules in beta-cells (Fig. 20C and G). The same conclusion can also be reached from experiments similar to those shown in Fig. 18. Raising the concentration of glucose from 3 to 15mM in the presence of tolbutamide augmented insulin secretion more than 4-fold in islets treated with cytochalasin B + taxol or with latrunculin B + nocodazole (not shown).

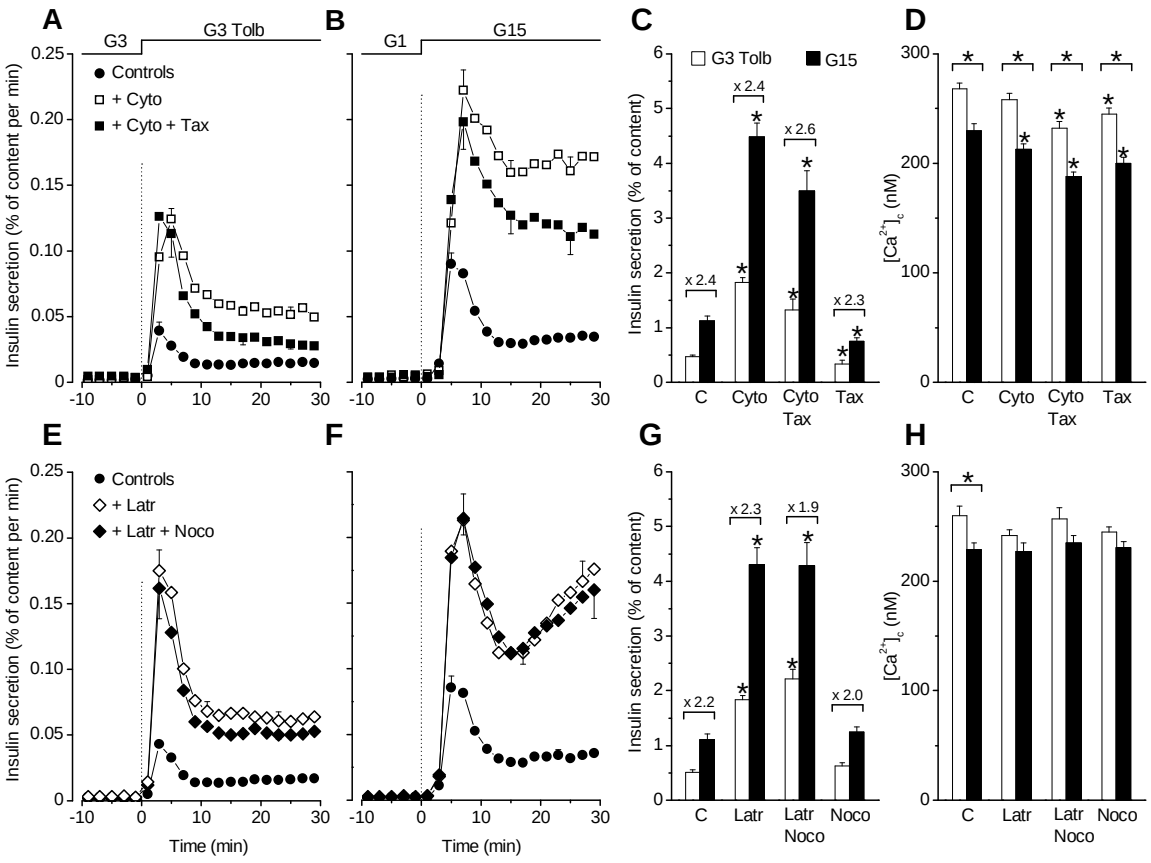

Fig. 20: Effects of combined inactivation of microtubules and microfilaments on glucose- and tolbutamide-induced insulin secretion. A-D: islets were first preincubated for 90 min with or without 10µM cytochalasin B (Cyto) and 5µM taxol. They were then perifused in the presence of the drugs to measure insulin secretion or $[Ca^{2+}]_c$. A and B: time course of insulin secretion changes induced by tolbutamide (Tolb) in 3mM glucose (G3) or by an increase in the glucose concentration from 1 to 15 mM (G15). C: insulin responses integrated over 30 min. D: $[Ca^{2+}]_c$ responses integrated over 30 min. For clarity, the effect of taxol alone is shown only as integrated changes; its time course was similar to that shown in Fig. 4. E-F: similar experiments using the combination of 10µM nocodazole (Noco) and 1µM latrunculin B (Latr). The only difference was that Latr was not present during the preincubation period with or without Noco, but added only 40 min before before stimulation with tolbutamide or glucose. * above a column indicates a significant difference ($P < 0.01$) with controls. Significant differences ($P<0.01$) between responses to Tolb in G3 (open columns) and to G15 (filled columns) are shown above pairs of columns, together with the fold-difference in insulin secretion (C and G).Values are means ± SE for 7-8 experiments of insulin secretion and 30-45 islets from 5-7 preparations (D) or 20-22 islets from 3 preparations (H) for $[Ca^{2+}]_c$.

2.2-DISCUSSION

The results of my second study show that beta-cell microtubules play little role in the short-term control of insulin secretion in mouse islets and, more specifically, are not implicated in metabolic amplification.

2.2.1-Experimental tools

Nocodazole completely disrupted islet microtubules but had virtually no effect on $[Ca^{2+}]_c$. Interestingly, nocodazole did not inhibit insulin secretion induced by three secretagogues, which suggests that previously observed inhibitions were at least partly secondary to changes in $[Ca^{2+}]_c$. We acknowledge however that nocodazole was reported to inhibit glucose-induced insulin secretion in rat islets (Howell and Tyhurst 1982) and HIT cells (Farshori and Goode 1994).

Although taxol is the most widely used agent to stabilize microtubules (Jordan and Kamath 2007), its effects on beta-cell function have received little attention. Addition of taxol simultaneously with high glucose did not affect insulin secretion in HIT cells (Farshori and Goode 1994), but pretreatment with the drug inhibited glucose-induced insulin secretion in rat islets (Howell and Tyhurst 1982). Here, we found that pretreatment with taxol completely stabilized microtubules in mouse islets, inhibited insulin secretion induced by KCl, tolbutamide or glucose, but also attenuated the rise in $[Ca^{2+}]_c$ produced by the three secretagogues. Microtubule-independent effects of taxol on Ca^{2+} release from the endoplasmic reticulum have been described in neural cells and cardiomyocytes (Boehmerle et al. 2007, Zhang et al. 2010), but we are not aware of effects on voltage-dependent Ca^{2+} channels. Whatever the underlying mechanism, the small decrease of $[Ca^{2+}]_c$ produced by taxol probably contributed to the partial inhibition of secretion.

2.2.2-Microtubules and metabolic amplification

The major aim of this study was to test the possibility that metabolic amplification of insulin secretion involves the microtubule system of beta-cells. Models implicating microtubules in metabolic amplification view the amplification process as an accelerated refilling of the pool of readily releasable granules through mobilization of insulin granules along microtubules (Eliasson et al. 2008, Varadi et al. 2002). Early cinematographic studies of primary beta-cells in monolayer cultures showed that glucose increased saltatory movements of insulin granules and that these movements were inhibited by vinblastine, vincristine or colchicine (Kanazawa et al. 1980, Lacy et al. 1975, Somers et al. 1979). More recent studies imaged cell lines in which insulin granules were tagged with a targeted fluorescent protein (Hao et al. 2005, Ivarsson et al. 2004, Varadi et al. 2003). This improved technique more clearly distinguished between slow random movements of the majority of granules and fast directed movements of a small population of granules. Stimulation of Ca^{2+} influx by either glucose or KCl selectively augmented directed granular movements, an effect that was suppressed by disruption of microtubules with nocodazole (Hao et al. 2005) or by inhibition of the microtubule-associated kinesin (Varadi et al. 2002, Varadi et al. 2003). These observations prompted the proposal that these fast, microtubule-dependent movements serve to replenish the readily releasable pool of granules (Hao et al. 2005) and that their acceleration by glucose corresponds to the amplification process (Varadi et al. 2002).

Our results refute the tested hypothesis. Amplification, defined as a larger secretion rate in the face of a similar or lower islet $[Ca^{2+}]_c$, was not impaired by disruption or stabilization of microtubules. Importantly, neither the magnitude nor the rapidity of the onset of amplification was altered by interference with microtubule function. Even when absolute levels of secretion were slightly reduced by taxol - an effect that we partly ascribe to inhibition of $[Ca^{2+}]_c$ - the relative change caused by amplification was unaffected. Metabolic amplification has long been regarded as a process that specifically contributes to sustain the second phase of

insulin secretion (Bratanova-Tochkova et al. 2002, Rorsman and Renstrom 2003, Straub and Sharp 2002, Varadi et al. 2003), but our recent data have shown that it is a rapid phenomenon that expresses itself within just a few minutes of exposure to high glucose (Henquin 2009, Henquin et al. 2006b, Mourad et al. 2010). This can be appreciated under control conditions (Fig. 19) where first phase insulin secretion is larger in response to glucose than to tolbutamide in the face of a lower triggering $[Ca^{2+}]_c$ signal. This can also be seen when the concentration of glucose is increased in the presence of a saturating concentration of tolbutamide (Fig. 18). Preservation of both the rapidity and magnitude of amplification after islet treatment with nocodazole and taxol implies that the process neither involves an acute action of glucose on microtubules nor is conditioned by a microtubule-mediated particular organization of pools of insulin granules.

2.3-GENERAL DISCUSSION OF STUDIES 1 AND 2

Rodent beta-cells contain 9000 – 13000 insulin granules (Dean 1973, Olofsson et al. 2002, Straub et al. 2004) distributed into geographically and/or functionally distinct pools. For the sake of simplicity, I will base the following discussion on a total of 10000 granules. About 50-100 granules are thought to constitute the RRP, a subset of the ~700 granules that are docked with the plasma membrane. The remaining ~9000 granules form the reserve pool, of which ~1500 are near, but not attached to, the plasma membrane (almost docked) (Olofsson et al. 2002, Straub et al. 2004). Current models ascribe the rapid first phase of insulin secretion to release of granules from the readily releasable pool, whereas the sustained second phase is thought to require the recruitment of granules from a reserve pool (Bratanova-Tochkova et al. 2002, Rorsman and Renstrom 2003). It is still debated whether this recruitment involves the physical mobilization of granules or a mere change in their properties (priming). The idea that granules must be docked before entering the releasable pool is also challenged by recent studies using the TIRF technique which

indicated that granules must not necessarily dock before entering the releasable pool and undergoing exocytosis ("newcomer granules") (Ohara-Imaizumi et al. 2007, Shibasaki et al. 2007, Izumi et al. 2007, Kasai et al. 2008).

From our measurements of insulin secretion rates and insulin content of the islets we can calculate, on the basis of 10000 granules per cell, the number of granules released by each beta-cell. In our control islets, we estimate that about 20 insulin granules were released per beta-cell during the first phase (7 min) of tolbutamide-induced insulin secretion, and about 30 granules during second phase (computed over 20 min) (Fig. 21A). During glucose-induced secretion, although $[Ca^{2+}]_c$ is lower than during tolbutamide stimulation, the numbers are 45 and 70 granules respectively (Fig. 21B). Although these numbers are smaller than the size of the readily releasable pool, the amplifying action of glucose (2-fold) is thus already evident during first phase. This remains true when first phase is repetitively triggered by four short pulses of high glucose (Fig. 14B). Such observations refute the idea that first phase is determined only by the amplitude of the triggering Ca^{2+} signal. A similar conclusion was reached previously by comparing the changes in $[Ca^{2+}]_c$ and insulin secretion in response to different glucose concentrations (Henquin et al. 2006b).

Disruption of the web of actin microfilaments by cytochalasin B or latrunculin B increases the number of insulin granules underneath the plasma membrane (VanObberghen et al. 1973, Jewell et al. 2008) and thus, presumably, the size of the readily releasable pool as in chromaffin cells (Vitale et al. 1995). This can explain how ~90 granules can be released during the first phase of tolbutamide-induced insulin secretion (Fig. 21A). Yet, the amplifying action of glucose on the first phase persists after latrunculin treatment (~140 granules), and does not fade out during triggering of several first phases (Fig. 14D). Obviously new granules can gain release competence within just a few minutes of glucose stimulation. We therefore propose that the amplifying effect of glucose during the first phase involves either a rapid action on the readily releasable pool or facilitation of the release of granules that come from another pool, both actions being independent of the operation of actin

filaments. The difference between granule numbers released per beta-cell in response to glucose or tolbutamide is even larger during the second phase (Fig. 21A and B). Importantly these marked differences persist in the absence of functional microtubules. The numbers of insulin granules released from islets treated with the combination of nocodazole and latrunculin are almost identical to those from islets treated with latrunculin alone (compare the numbers in Fig. 21A and B to those in Fig. 21C and D). Altogether, our results obtained in whole islets indicate that intact microtubules are not required for acute refilling of the releasable pool and for the short-term control of insulin secretion.

Interestingly, estimations of exocytosis by capacitance measurements in mouse beta-cells treated with colcemid (Proks and Ashcroft 1995) or INS1 cells treated with vindesin (Ivarsson et al. 2004) also suggested that microtubules are not necessary for replenishment of the readily releasable pool of insulin granules after its emptying by trains of depolarizations. We can also exclude that the microtubule-dependent acceleration of fast granular movements by glucose (Hao et al. 2005, Varadi et al. 2002) underlies metabolic amplification for a simple reason. Unlike insulin secretion (Fig. 17) (Henquin 2000), this acceleration of granular movements was not augmented further by high glucose in the presence of KCl (Hao et al. 2005). Kinesin-driven translocation of insulin granules along microtubules (Donelan et al. 2002, Meng et al. 1997, Varadi et al. 2002) probably contributes towards the maintenance of long-term beta-cell secretory function, but is unlikely to play an active role in the acute regulation of insulin secretion, except perhaps in pathological degranulated beta-cells or in inherently poorly granulated cell lines. Other functions of kinesin-1 are also possible. Thus, the targeted inactivation of kinesin-1 in beta-cells led to the inhibition of both phases of glucose-induced insulin secretion *in vivo*, independently of any alterations in sub-cellular localization of insulin granules (Cui et al. 2011).

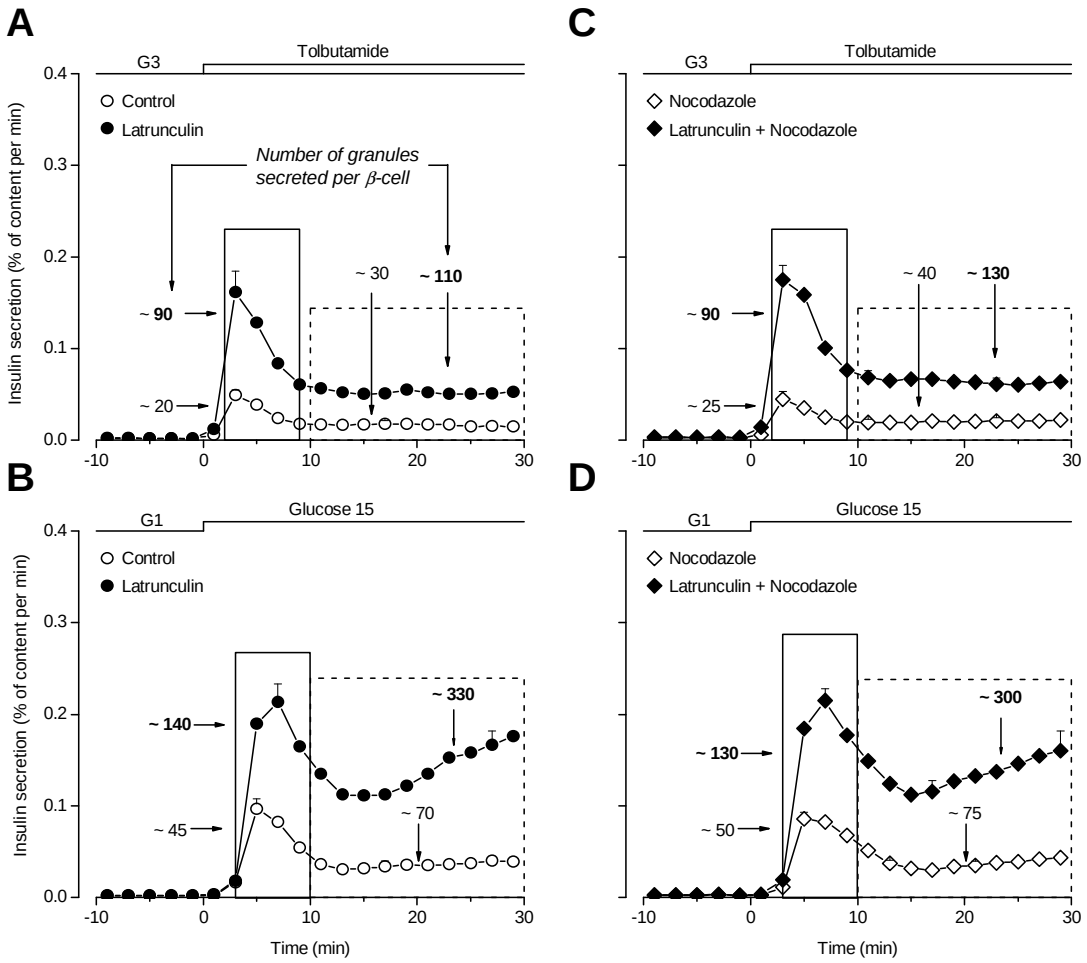

Fig. 21: Estimation of the number of granules released per individual β-cell during the two phases of insulin secretion stimulated by tolbutamide (A and C) or glucose (B and D) in control islets and islets treated with latrunculin B, nocodazole or a combination of both. Calculations are based on actual rates of secretion and insulin contents of the islets. For facility, we used the numbers of 10000 granules per β-cell in control islets and 9000 per β-cell in islets treated with latrunculin B (because the islet content was ~10% lower). First phase was integrated over 7 min (2-9 min for tolbutamide and 3-10 min for glucose) and second phase was integrated over 20 min (10-30 min for both).

Straub and Sharp (2004) have proposed a model in which the releasable pool itself contains a smaller pool of *immediately* releasable granules (with full competence), and suggested that the rate-limiting step of secretion is entry into that smaller pool. In their model, amplification corresponds to the conversion of docked granules into immediately releasable granules. Our results are compatible with their proposal that amplification corresponds to acquisition of full release competence by the granules. However, we cannot distinguish between the two possibilities: acceleration of the priming process of previously docked granules or facilitation of exocytosis of previously undocked granules. Straub and Sharp also proposed that the size of first phase is dependent on the size of the immediately releasable pool, which can be influenced by the "history of exposure to glucose", i.e. a time-dependent potentiation mediated by the amplifying pathway (Straub and Sharp 2004). Our interpretation is slightly different. Thus, we show that metabolic amplification is a rapid phenomenon that is turned on almost as rapidly as the production of the triggering signal and thereby controls the amplitude of first phase. Our observations also call for amendments to mathematical models of the amplifying pathway (Bertuzzi et al. 2007, Chen et al. 2008).

Several recent studies using total internal reflection fluorescence microscopy to visualize exocytotic events indicate that newcomer granules (i.e. granules that belong to neither the readily releasable nor the docked pool) are being released during both the first and second phases (Izumi et al. 2007, Shibasaki et al. 2007, Kasai et al. 2008) or preferentially during second phase (Ohara-Imaizumi et al. 2007) of glucose-induced insulin secretion. An interesting model (Pedersen and Sherman 2009) has proposed that these newcomer granules correspond to a functional pool of highly Ca^{2+}-sensitive granules, which can be increased by glucose (Yang and Gillis 2004). In the light of these results and of the current study, we would like to speculate that amplification corresponds to facilitation of release of these newcomer granules.

In conclusion, amplification of insulin secretion by glucose is rapid and, contrary to current models, influences the first as well as the second phase through cytoskeleton-independent mechanisms. Because the process corresponds to the release of larger numbers of granules for similar or lower $[Ca^{2+}]_c$ in beta-cells (Henquin 2000), our laboratory previously suggested that an increase in the efficacy of Ca^{2+} on exocytosis is the underlying cause. We would now rather suggest that metabolic amplification augments the number of release-competent insulin granules, possibly in a highly Ca^{2+}-sensitive pool. This should now help identifying the molecular mechanisms and cellular effectors of a quantitatively important (Henquin 2009) regulatory step of stimulus-secretion coupling in beta-cells.

Study 3

Involvement of AMPK in metabolic amplification of insulin secretion

Unpublished results

AMPK functions as a sensor of cellular energy status (Hardie 2008). In pancreatic beta-cells, AMPK activity can be regulated by glucose through changes in adenine nucleotides. Thus, when beta-cell oxidative metabolism is enhanced by increasing glucose concentrations, the resulting rise in ATP and decrease in ADP and AMP concentrations leads to a decrease in AMPK activity (Rutter et al. 2003). AMPK might thus be an effector in fuel-induced insulin secretion, but the available data are contradictory. My aim in this short part of my thesis work was to evaluate the involvement AMPK in insulin secretion and particularly in metabolic amplification.

3.1-AMPK subunits isoforms

There exist multiple isoforms of each of the 3 subunits composing AMPK (α_1, α_2; β_1, β_2; γ_1, γ_2, γ_3). Different combinations of the isoforms are expressed in a tissue-specific manner: complexes containing α_2 predominate in muscles while hepatocytes display equal amounts of α_1 and α_2 activity. The main catalytic subunit isoform expressed in pancreatic beta-cells is α_1 (Rutter et al. 2003) and expression level of the α_2-subunit is 10 times less. It has also been shown that the α_1-subunit is mostly cytosolic whereas the α_2-subunit is concentrated in the perinuclear region (Sun et al. 2010). The β_1-subunit seems to be preferentially found in complexes containing α_1, whereas β_2 is often associated with α_2-containing trimers (Scott et al. 2008).

3.2-AMPK activators

AMPK activation requires phosphorylation of Thr172 in the activation loop of the catalytic α-subunits by the upstream LKB1 or CamKKβ. Several classes of AMPK activators exist. One of these pharmacological agents, the biguanide metformin, has long been used to treat type 2 diabetes. It acts by reducing blood glucose levels via AMPK-dependent inhibition of hepatic gluconeogenesis. Like the related phenformin, metformin has no direct effect on AMPK activity. Both compounds act by inhibiting complex I of the mitochondrial respiratory chain resulting in an increase in AMP/ATP ratio (Fogarty and Hardie 2010).

5-aminoimidazole-4-carboxamide riboside (AICAR) is widely used to investigate the downstream effects of AMPK activation. In cells, AICAR is converted by adenosine kinase to ZMP, an analog of AMP which can bind to the γ subunit and activate AMPK (Fogarty and Hardie 2010). However, it has been previously suggested that AICAR metabolism in beta-cells leads to sequential generation of ZMP, ZDP and ZTP (Malaisse et al. 1994, Rutter et al. 2003) which might generate off-target effects.

The thienopyridone compound A-769662 acts as a direct allosteric activator of AMPK by binding to the β1 subunit (Scott et al. 2008) as well as by inhibiting Thr172 dephosphorylation. This novel drug has the advantage of not affecting adenine nucleotides and having fewer off-target effects than other activators. *In vivo* studies have shown that A-769662, by activating AMPK, lowers plasma glucose by 40%, reduces body weigh gain and decreases both plasma and liver triglyceride levels in ob/ob mice (Cool et al. 2006).

3.3-AMPK activation in mouse islets

To study the involvement of AMPK in insulin secretion, I used these 3 activators. Their efficiency in activating islet AMPK was assessed by measuring phosphorylation of acetyl CoA carboxylase (ACC), its best known downstream

target (Leclerc et al. 2004, Kefas et al. 2004, Gleason et al. 2007). Physiologically, phosphorylation of ACC1 and ACC2 inactivates both isoforms, inhibits fatty acid synthesis and promotes fatty acid oxidation (Fogarty and Hardie 2010).

As shown in figure 22, phosphorylated ACC was detected in control islets incubated in the presence of glucose alone. Metformin increased ACC phosphorylation (Fig. 22, 2nd lane), as did A-769662 (Fig. 22, 4th lane). Unexpectedly, AICAR treatment failed to increase ACC phophorylation and even decreased it (Fig. 22, 3rd lane). This was also true when a higher concentration (1 mM) of AICAR was used. These results suggest that, as expected, metformin and A-769662 increase AMPK activity in isolated mouse islets under our experimental conditions. Surprisingly, and in contrast with previous findings in MIN6 cells (Da Silva Xavier et al. 2003), we did not detect any activation of AMPK after treating mouse islets with 0.5-1.0 mM AICAR.

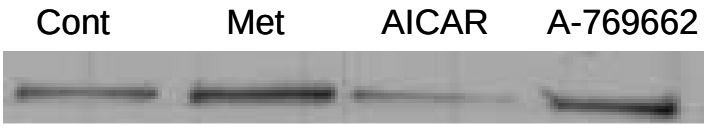

Fig. 22: Assay of AMPK activation by detection of Acetyl CoA Carboxylase (ACC) phosphorylation.
Batches of 70 islets were incubated for 60 min in a Krebs buffer containing 15 mM glucose ± 100 µM Metformin, 500 µM AICAR or 20 µM A-769662. An anti-phospho-ACC antibody was then used to detect phosphorylated ACC in islet lysates submitted to western blotting. These blots are representative of two similar experiments.

3.4-Effects of AMPK activators on glucose-induced insulin secretion

A possible influence of AMPK activity on insulin secretion was suggested by experiments using pharmacological activators or genetic up/down regulation of AMPK in beta-cells. However, the published results are controversial. Studies by Rutter and colleagues reported impaired insulin secretion upon AMPK activation with AICAR (Da Silva Xavier et al. 2003), metformin (Kefas et al. 2004, Leclerc et

al. 2004) or overexpression of a constitutively active form of the enzyme (Da Silva Xavier et al. 2003). Knock-out of both α_1 and α_2 subunits isoforms of AMPK resulted in decreased insulin secretion *in vivo* and increased glucose-induced secretion *in vitro* (Sun et al. 2010).

By contrast with these findings, AICAR and phenformin-induced activation of AMPK did not antagonize glucose-induced insulin secretion, and expression of a dominant negative form of AMPK had no effect both tested in MIN6 cells (Gleason et al. 2007). In fact, AICAR was previously reported to increase glucose-stimulated insulin secretion from rat islets (Akkan and Malaisse 1994), and these observations have recently been confirmed in mouse islets (Düfer et al. 2010). These discrepancies may be attributed to different experimental models as well as direct or indirect inhibitory effects of AICAR on K_{ATP} channels. It was thus shown that AICAR decreases K-ATP channels activity, depolarizes the membrane potential and increases $[Ca^{2+}]_c$ (Düfer et al. 2010). The role played by AMPK in glucose-induced insulin secretion thus remains debated.

To assess the influence of AMPK on insulin secretion, I used the islet perifusion method described earlier. 100µM Metformin or 500µM AICAR were added to the perifusion media in test groups and insulin secretion was stimulated by increasing the glucose concentration from 1 to 15mM. The control biphasic secretory response is shown by open circles in figure 23A and B. Metformin decreased (Fig. 23A), whereas AICAR increased (Fig. 23B) glucose-induced insulin secretion, mainly during the second phase. These observations contrast with the inhibitory effect of AICAR reported by the group of Rutter (Da Silva Xavier et al. 2003, Kefas et al. 2004, Leclerc et al. 2004) but are in agreement with other studies (Akkan and Malaisse 1994, Dufer et al. 2010).

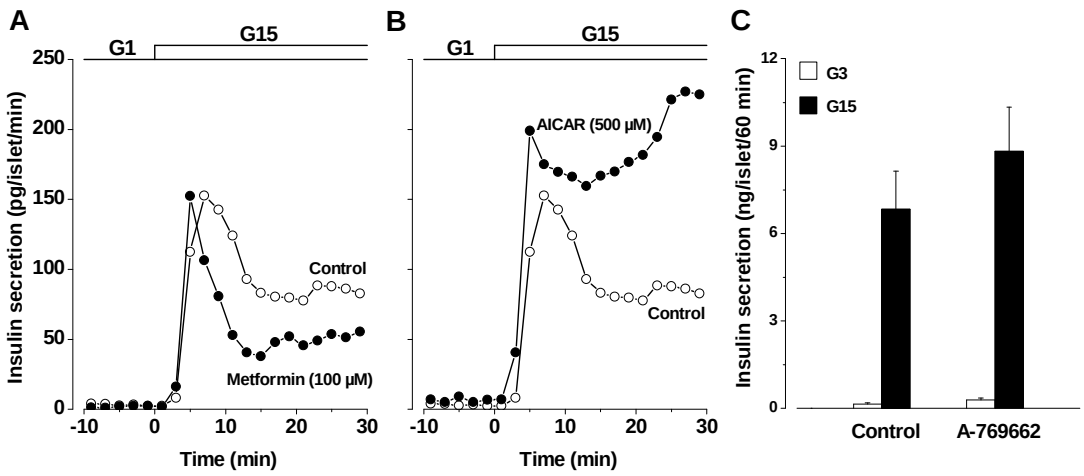

Fig. 23: Effect of AMPK activators on glucose-induced insulin secretion.
A and B: insulin secretion in response to 15 mM glucose (G15) measured in control islets (open circles) perifused without or test islets perifused in the presence of 100 µM Metformin (A) or 500 µM AICAR (B). C: Glucose-induced insulin secretion during static incubations in the presence of 3 mM (open columns) or 15 mM (black columns) glucose. 20 µM of the direct AMPK activator compound A-769662 were added to test media when appropriate. Results are mean values of 2 experiments in A and B, and means ± SEM for 6 batches of islets in C.

Because of these conflicting results obtained using indirect AMPK activators and taking into consideration the lack of effect of AICAR on AMPK activity in my preparations, I used A-769662 to test the effects of direct and more specific AMPK activation on insulin secretion. Because of limited availability of the substance, I used a system of islet incubation. As shown in figure 23C, A-769962 had no significant impact on either basal insulin secretion or the response to 15 mM glucose.

In summary, my results show that while AMPK activation by metformin indeed decreases the secretory response to glucose, the more specific and direct AMPK activator A-769662 does not affect insulin secretion. In my experiments, AICAR increased the insulin response to glucose without increasing AMPK activity. This observation corroborates existing doubts regarding the specificity of this drug and indicates that its effects on insulin secretion might actually be due to its action on K_{ATP} channels via the ATP analog ZTP produced by AICAR metabolism in the cell (Dufer et al. 2010).

3.5-Effect of AMPK activators on metabolic amplification

It has been proposed that AMPK affects distal steps of insulin secretion beyond the Ca^{2+} triggering signal. This suggestion was based on experiments where expression of a constitutively active form of AMPK in MIN6 cells inhibited glucose-induced vesicle movements and decreased the number of docked and fusing granules independent of changes in $[Ca^{2+}]_c$ and ATP (Tsuboi et al. 2003). This study also showed that the decrease in glucose-induced granule movement and exocytosis upon forced AMPK activation was associated with failure of glucose to induce rearrangement of the cortical F-actin web (Tsuboi et al. 2003). In 2009, the same group amended their first interpretations. The authors then showed that glucose did not increase overall granule movements but increased the number of granules undertaking long excursions at speeds in the order of several µm/s (Hao et al. 2005). Their working hypothesis was that AMPK action involves cytoskeleton-related motor proteins such as kinesin which can be phosphorylated by AMPK and to affect granule movement toward the cell periphery (McDonald et al. 2009). In contradiction with their early results, AMPK activation did not affect granule movement and did not modify the phosphorylation state of kinesin light chain-1 (KLC1) thought to be involved in granule movements along microtubules (McDonald et al. 2009).

To test the involvement of AMPK in metabolic amplification of insulin secretion, I compared the secretory responses to 30mM KCl in low vs. high glucose in control islets and in the presence of different AMPK activators. As shown in figure 24A, K30-induced insulin secretion is much greater in the presence of 15mM than 3mM glucose. The difference in the secretory responses is attributable to the amplifying effect of glucose as detailed earlier.

In the presence of 100µM metformin (Fig. 24B), the secretory response was unaffected in low glucose but amplification was completely suppressed. The presence of 500µM AICAR, had no effect on K30-induced insulin secretion at low or high glucose. Finally, direct AMPK activation by A-769662 (20µM) slightly increased K30-induced insulin secretion during static incubation experiments (Fig.

24D). This increase was not significant in low glucose but reached statistical significance in high glucose. The difference between the responses in low vs. high glucose i.e. metabolic amplification was not affected by the drug. In our experiments, metformin specifically attenuated depolarization-evoked secretion in the presence of

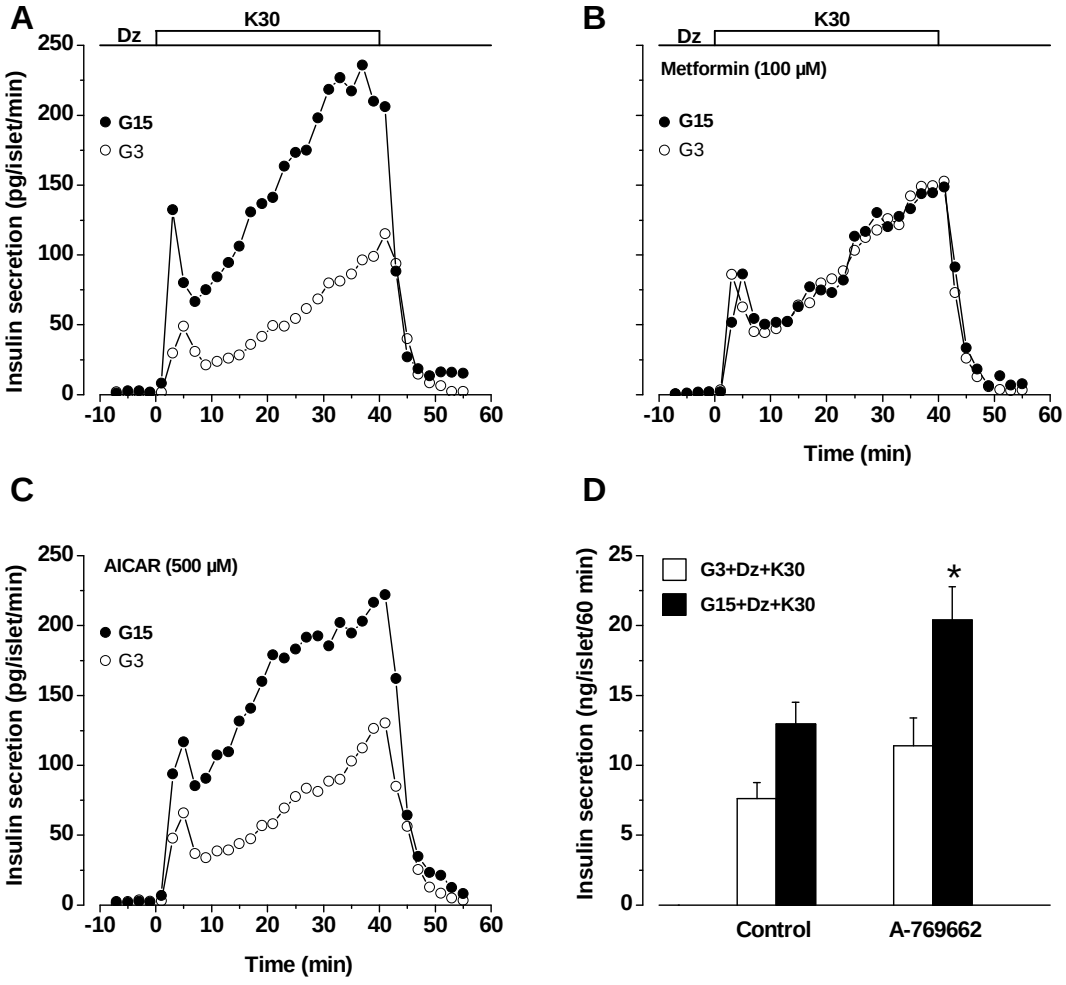

Fig. 24: Effect of AMPK activators on K30-induced insulin secretion and metabolic amplification.
A-C: K30-induced insulin secretion in the presence of 100 µM diazoxide (Dz) and either 3 mM glucose (G3; open circles) or 15 mM glucose (G15; filled circles). Secretion was measured during perifusion experiments using control islets (A) or in the presence of 100 µM Metformin (B) or 500 µM AICAR (C). D: K30-induced insulin secretion during static incubations in the presence of 100 µM diazoxide and either 3 mM (white columns) or 15 mM (black columns) glucose. 20 µM of the direct AMPK activator compound A-769662 were added to test media. Results are mean values of 2 experiments in A-C and means ± SEM for 6 batches of islets in D.

high glucose. Since this drug probably inhibits mitochondrial ATP synthesis, I speculate that metformin affects metabolic amplification by lowering cytosolic ATP rather than by activating AMPK. ATP is indeed a major candidate as a mediator of amplification as we have seen in the introduction. That the effect is not due to AMPK activation is shown by our observation that direct, more specific activation of AMPK by A-769662 does not decrease the secretory response to K30 and does not attenuate amplification of insulin secretion by glucose.

To understand the role of AMPK in metabolic amplification of insulin secretion despite the relative inconsistency of results obtained using pharmacological activators, I planned studying islets from AMPKα_1 and/or α_2-null mice. Unfortunately, such mice were not made available to us. I only could perform a single series of experiments (n=1) using islets from one AMPKα_1-null mouse. The results showed that metabolic amplification persisted in the absence of AMPK activity (data not shown).

3.6-Conclusions

In these preliminary and admittedly incomplete experiments, I did not obtain any evidence suggesting that AMPK is involved in acute control of insulin secretion, particularly in metabolic amplification. This part of my project was thus stopped.

Study 4

Role of actin microfilaments in neurohormonal amplification of insulin secretion: the cAMP pathway

Unpublished results

4.1-INTRODUCTION

4.1.1-Insulin secretion and cAMP

That cAMP increases insulin secretion has long been established by experiments using exogenous cAMP, direct activation of adenylyl-cyclase with forskolin or inhibition of phosphodiesterases by methylxanthines (reviewed by Malaisse and Malaisse-Lagae 1984, Henquin 1985, Prentki and Matschinsky 1987). Several isoforms of adenylyl-cyclases and phosphodiesterases are expressed in beta-cells (Ramos et al. 2008, Furman et al. 2010) and mediate the effects of distinct agents. Physiologically, the incretins GLP-1 and GIP potentiate insulin secretion via activation of G-protein-coupled adenylyl cyclase, which causes a rapid elevation of beta-cell cAMP (Gromada et al. 1998, Ramos et al. 2008, Leech et al. 2011). Incretins amplify insulin secretion induced by elevated glucose levels during meals. Glucagon can also increase insulin secretion but does not do so during fasting periods because beta-cells are inhibited by the low ambient glucose levels.

A direct involvement of cAMP in the signaling pathway of glucose-induced insulin secretion is more controversial because the available results differ depending on the model and the species. Thus, glucose was found to increase cAMP content in rat islets (Grill and Cerasi 1973, Sharp 1979) but not in purified rat beta-cells (Schuit and Pipeleers 1985) or mouse islets (Thams et al. 1982, Henquin and Meissner 1984). When it has an effect, glucose causes a delayed rise cAMP rise attributed to activation of a Ca^{2+} and ATP-sensitive soluble adenylyl-cyclase.

Recent studies succeeded in measuring cAMP in the submembrane space of mouse beta-cells. cAMP was found to vary with an oscillatory pattern in response to GLP-1 (Dyachok et al. 2006) and to glucose (Dyachok et al. 2008). Moreover, glucose-induced cAMP oscillations were closely linked to $[Ca^{2+}]_c$ oscillations, both being suppressed in the absence of extracellular Ca^{2+} or when L-type Ca^{2+} channels were inhibited (Dyachok et al. 2008). The interplay between cAMP and $[Ca^{2+}]_c$ appeared to involve both Ca^{2+} effects on cAMP dynamics via regulation of adenylyl cyclase and phosphodiesterase (Landa et al. 2005, Dyachok et al. 2008) and cAMP effects on the Ca^{2+} signal via regulation of K-ATP channels (Kang et al. 2008), voltage-dependent Ca^{2+} entry (Ammala et al. 1993a) and Ca^{2+}-induced Ca^{2+} release (Kang et al. 2003). Such a coordination of glucose-induced oscillations of cAMP and $[Ca^{2+}]_c$ was found to be required for optimal amplitude of pulsatile insulin release from single, isolated beta-cells (Dyachok et al. 2008).

4.1.2-The effectors of cAMP in insulin secretion

cAMP increases insulin secretion through two distinct pathways:

- by binding to the regulatory subunit of protein kinase A (PKA), cAMP causes dissociation of the active catalytic subunits which phosphorylate a number of incompletely identified target proteins (Jones and Persaud 1998).
- by activating Epac2 (Exchange protein directly activated by cAMP), also known as cAMP-regulated protein guanine nucleotide exchange factor (cAMP-GEFII), cAMP exerts rapid PKA-independent effects (Eliasson et al. 2003, Holz et al. 2004).

The PKA pathway: Activation of PKA leads to phosphorylation of several proteins in beta-cells, but how this phosphorylation results in an increase of insulin secretion is not fully understood.

Glucose transporter-2 (Glut2), the Kir6.2 and SUR1 subunits of K-ATP channels are phosphorylated by PKA (Yu and Jin 2010), as well as proteins

associated with the secretory process (Seino et al. 2009). Among the latter, Rac1, a member of the Rho GTPase family, was found to be activated by forskolin and to translocate to the plasma membrane in INS1 cells where it might regulate actin polymeriztion (Li et al. 2004). It is thus plausible that cAMP activates Rac1 via PKA to facilitate the trafficking of secretory granules in beta-cells through formation of F-actin. This sequence of events is invoked by the inhibition of forskolin-stimulated insulin secretion and the disappearance of F-actin filaments in cells expressing a dominant-negative Rac1 (Li et al. 2004). The above data are in agreement with earlier suggestions that PKA activation increases the number of readily releasable granules (Renstrom et al. 1997) and of highly Ca^{2+}-sensitive granules (Wan et al. 2004), although some of the effects of cAMP on granule availability have been found to be PKA-independent (Eliasson et al. 2003). Interestingly, the increase in size of the pool of highly Ca^{2+}-sensitive granules induced by PKA is additive with the increase induced by glucose, suggesting that the effect of glucose is cAMP/PKA-independent. In contrast, the effects of PKA and PKC (discussed later) on pool size are not additive, which suggests that activation of the two kinases converges on a common mechanism (Yang and Gillis 2004, Wan et al. 2004). That such effects of cAMP/PKA on insulin granule availability for release could involve the cortical F-actin network is suggested by experiments showing that forskolin reduces F-actin staining and increases the number of granules morphologically located beneath the plasma membrane, two effects mimicked by actin depolymerization with latrunculin (Pigeau et al. 2009).

The Epac2 pathway: Implication of a PKA-independent pathway in the potentiation of insulin secretion by cAMP was suggested after observations that PKA inhibition did not completely suppress the effects of cAMP on exocytosis (Renstrom et al. 1997). This second pathway involves the cAMP-binding protein Epac2, a cAMP sensor that seems to mediate the increase in fusion events elicited by the addition of exogenous cAMP during glucose stimulation (Shibasaki et al. 2007, Seino et al. 2009).

The subcellular localization of Epac2 splice variants determines their involvement or not in the secretory process. Epac2A localized near the plasma membrane thus contributes to glucose + cAMP-induced insulin secretion in MIN6 cells whereas Epac2B, found in the cytosol, does not (Seino et al. 2009). The small GTPase Rap1 may be the effector of this Epac2-dependent pathway by increasing the number of restless newcomer granules particularly during the first phase of glucose-induced insulin secretion. Utilization of Epac2-specific analogs of cAMP in mouse islets also resulted in increased glucose-induced insulin secretion during both first and second phases (Kelley et al. 2009).

In addition to these effects on the final steps of granule exocytosis, Epac2 is thought to affect secretion by inhibiting K-ATP channel activity and raising $[Ca^{2+}]_c$ via increased Ca^{2+} influx and mobilization of intracellular Ca^{2+} (Kang et al. 2005, 2006, Liu et al. 2006).

4.1.3-Role of cAMP in the metabolic amplification of insulin secretion

The proposal that cAMP mediates the amplifying effect of glucose is based upon the notion that glucose increases beta-cell cAMP (Landa et al. 2005, Dyachock et al 2008), at least in the submembrane region, and that cAMP can increase the secretory response without changing $[Ca^{2+}]_c$. The second property has been established in permeabilized islets incubated in the presence of a fixed $[Ca^{2+}]_c$ (Tamagawa et al. 1985, Jones et al. 1986), and confirmed at the single beta-cell level, in which insulin granule exocytosis was estimated as changes in membrane capacitance (Renstrom et al. 1997). In single beta-cells studied using two-photon excitation imaging, cAMP was found to increase Ca^{2+}-induced exocytosis in the presence of high but not low glucose, which led the authors to conclude that a factor other than cAMP is also needed (Hatakeyama et al. 2006). Other observations also argue against the exclusive role of cAMP. Thus, glucose could increase KCl-induced insulin secretion even when cAMP was raised to very high levels (Gembal et al. 1993, Sato and Henquin 1998), and amplification persisted when PKA was inhibited

(Harris et al. 1997, Sato and Henquin 1998). In addition, there was no correlation between insulin secretion and total cAMP concentrations in mouse islets (Sato and Henquin 1998).

Our laboratory has thus favored the involvement of cAMP in hormonal amplification but not in the metabolic amplifying pathway of glucose. However, because recent reports suggested that glucose-induced oscillations of cAMP in the submembrane region (Dyachok et al. 2008) are important for glucose-induced insulin secretion (Idevall-Hagren et al. 2010), a re-evaluation of the role of cAMP in metabolic amplification seems appropriate.

4.1.4- Role of microfilaments in cAMP-mediated increase in insulin secretion

I have described above a number of observations that are compatible with an action of cAMP on the network of beta-cell microfilaments. However, the implication of microfilaments in the increased insulin secretion produced by cAMP was rarely tested directly. One study comparing the potentiating actions of cytochalasin B and theophylline (a phosphodiesterase inhibitor) in the perfused rat pancreas found them to be additive (VanObberghen et al. 1975), whereas no additivity was observed by another laboratory using purified rat beta-cells (Wang et al 1990). The question remains open.

4.2-PERSONAL RESULTS

The aim of my study was two-fold: to investigate whether the polymerization state of beta-cell actin microfilaments is important for cAMP amplification of glucose-induced insulin secretion, and to reevaluate the possibility that cAMP is involved in metabolic amplification. The experiments were performed with mouse islets treated with latrunculin to depolymerize actin and jasplakinolide to polymerize it. cAMP levels were increased by a combination of 1µM forskolin to activate adenylyl-cyclase and 10µM isobutyl-methylxanthine (IBMX) to inhibit phosphodiesterases.

4.2.1-Impact of microfilament disruption and stabilization on cAMP-dependent amplification of glucose-induced insulin secretion

In a first series of experiments, islets were exposed to IBMX + forskolin during stimulation with 15 mM glucose (Fig. 25: between 0 and 40 min). In control islets, this caused a ~8-fold increase in secretion that was reversible upon wash-out of the two drugs. When similar experiments were carried out in the presence of 1µM latrunculin, glucose-induced insulin secretion was increased ~4-fold compared to controls, as expected (Mourad et al. 2010), and cAMP elevation by IBMX and forskolin caused a further ~4-fold increase in secretion (Fig. 25). The effects of actin depolymerization and cAMP elevation on secretion thus appear to be additive. Disruption of microfilaments does not impair the amplifying action of cAMP. A third group of islets was pre-treated with 1µM jasplakinolide to cause actin polymerization prior to perifusion experiments. I have previously shown that the effects of jasplakinolide on actin polymerization are not reversible upon removal of the drug (Mourad et al. 2010). Actin polymerization increased the secretory response to glucose ~2-fold, and cAMP augmented it to similar levels as in controls (Fig. 25).

The involvement of actin microfilaments in cAMP-mediated amplification of insulin secretion was also investigated using another protocol. Untreated islets were perifused in 15 mM glucose before being acutely exposed to either latrunculin or jasplakinolide. As shown in Figure 26A, latrunculin caused a progressive but large (~5-fold) and reversible increase in glucose-induced insulin secretion. When the experiment was repeated in the presence of IBMX and forskolin, the secretory response to 15mM glucose was enhanced ~6-fold, but actin depolymerization by latrunculin caused a further 2-fold increase (Fig. 26B). Acute exposure of control islets to jasplakinolide caused a progressive increase of glucose-induced secretion that persisted after washing of the drug, thus confirming our earlier observations of the irreversibility of jasplakinolide effects (Mourad et al. 2010). Interestingly, when jasplakinolide was added in the presence of IBMX and forskolin, the drug did not increase secretion beyond than the increase caused by cAMP elevation (Fig. 26B) suggesting non-additive effects of actin polymerization and cAMP elevation.

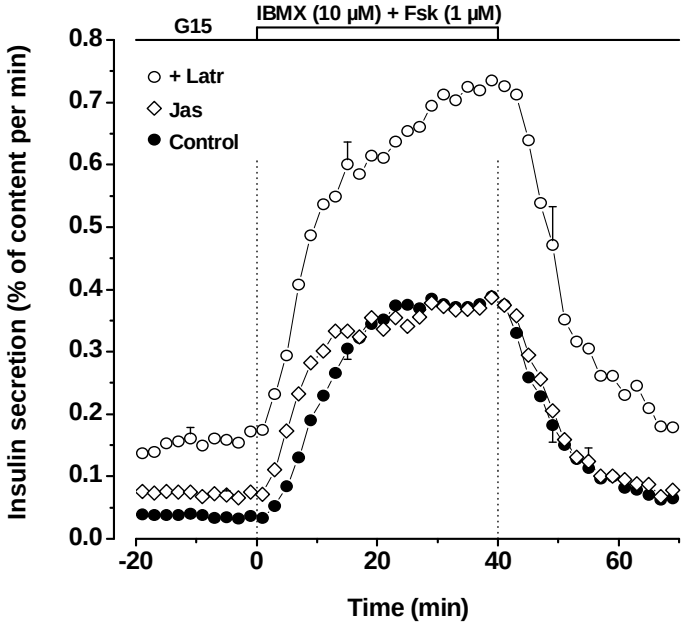

Fig. 25: Effect of actin depolymerization and polymerization on cAMP-dependent increase of glucose-induced insulin secretion. Islets were stimulated with 15 mM glucose (G15) at -40 min and a combination of IBMX (10 µM) and forskolin (Fsk; 1 µM) was added between 0 and 40 min. In one group (o), 1 µM latrunculin (Latr) was present throughout. In another group (◊), the islets were preincubated for 90 min in the presence of 1 µM jasplakinolide (Jasp), but the drug was not added to perifusion media. Values are means ± SE for 6-7 experiments.

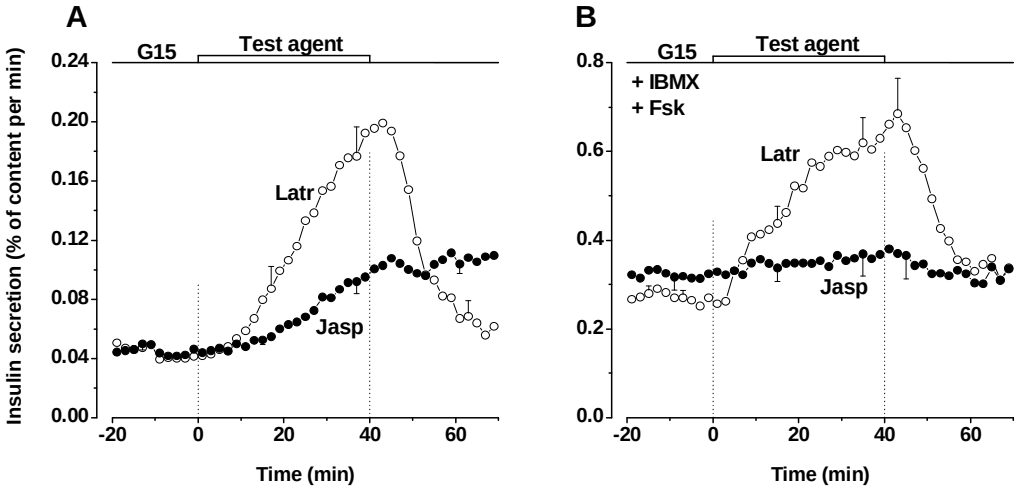

Fig. 26: Effects of actin depolymerization and polymerization on insulin secretion induced by glucose alone or in combination with IBMX and forskolin. A: Mouse islets were stimulated with 15 mM glucose (G15) at -40 min. 1 µM latrunculin (Latr; open circles) or 0.5 µM jasplakinolide (Jas; filled circles) was added between 0 and 40 min. B: Experiments similar to those shown in A were done in the presence of IBMX (10 µM) and forskolin (Fsk; 1 µM) throughout. Note the difference in scale between A and B. Values are means ± SE for 6 (Latr) or 5 (Jasp) experiments.

4.2.2-Involvment of cAMP in the metabolic amplification of insulin secretion

The experiments which allowed the laboratory to previously conclude that cAMP is not involved in metabolic amplification used the approach of $[Ca^{2+}]_c$ clamping by high KCl in the presence of diazoxide (Gembal et al. 1993, Sato and Henquin 1998). To retest the hypothesis, I chose to compare $[Ca^{2+}]_c$ and insulin responses in islets stimulated with glucose or tolbutamide, in the presence or absence of IBMX + forskolin.

Figure 27A shows that, again in this series, the increase in islet $[Ca^{2+}]_c$ was larger in response to 500µM tolbutamide + 3mM glucose than to 15mM glucose, the difference being significant during first and second phases (Fig. 27G and H). cAMP elevation did not affect basal $[Ca^{2+}]_c$ levels nor did it affect the response to glucose in terms of amplitude (Fig. 27D, G, H). There was, however, a striking modification in the response of $[Ca^{2+}]_c$ to glucose. The oscillations observed during the second phase in control islets (Fig. 27B shows a representative trace) were abolished when cAMP was elevated, and were replaced by continuous rapid spiking (Fig. 27E, representative trace). IBMX + forskolin did not affect the average $[Ca^{2+}]_c$ response to tolbutamide during the first phase but slightly increased it during second phase (Fig. 27D, G, H), without significantly changing the pattern of individual responses (Fig. 27C and F).

Next, we studied insulin secretion under the same experimental conditions (Fig 28). In controls, the secretory response to glucose was 2-fold higher than to tolbutamide during both the first and second phases of secretion (Fig. 28A, G, H). Microfilament depolymerization with latrunculin (Fig. 28B) and stabilization with jasplakinolide (Fig. 28C) similarly increased the secretory response to both tolbutamide and glucose, so that the amplifying effect of glucose persisted during the first (Fig. 28G) and second (Fig. 28H) phases, as observed in a previous study (Mourad et al. 2010).

As compared with controls (Fig. 28A), IBMX and forskolin increased the secretory response to tolbutamide ~6-fold during first phase and ~10-fold during second phase (Fig 28D, note the differences of scales). Interestingly, the response to glucose was increased only ~3-fold during the first phase and ~9-fold during the second (Fig. 28D, G, H). As a result of the greater amplification of the response to tolbutamide during the first phase, metabolic amplification, i.e. the difference between responses to glucose and tolbutamide, vanished during first phase (Fig. 28G) but remained unaffected during the second phase (Fig. 28H). Microfilament depolymerization with latrunculin or stabilization with jasplakinolide did not alter cAMP amplification of insulin secretion during stimulation with glucose or tolbutamide (Fig. 28E, G, H). However, compared with untreated control islets, the amplification by cAMP was augmented by latrunculin and unchanged by jasplakinolide (Fig. 28F, G and H). Importantly, the suppression of metabolic amplification by cAMP during the first phase and its persistence during the second phase were observed independently of the state of actin microfilaments.

4.3-DISCUSSION

The results presented above establish that actin microfilaments are not required for cAMP to amplify glucose- or tolbutamide-induced insulin secretion. They also show that metabolic amplification of the first phase of insulin secretion may involve cAMP-dependent mechanisms whereas the second phase does not seem to require such mechanisms.

Recordings with intracellular microelectrodes have shown that cAMP increases glucose- or tolbutamide-induced Ca^{2+}-dependent electrical activity in beta-cells within intact mouse islets (Henquin and Meissner 1984, Henquin et al. 1987). These observations prompted the suggestion that cAMP promotes Ca^{2+} influx in beta-cells, an effect that could be mediated by changes in the properties of voltage-dependent Ca^{2+} channels (Ammala et al. 1993b) or K-ATP channels (Kang et al. 2008). I show here that $[Ca^{2+}]_c$ is indeed increased by cAMP during glucose or

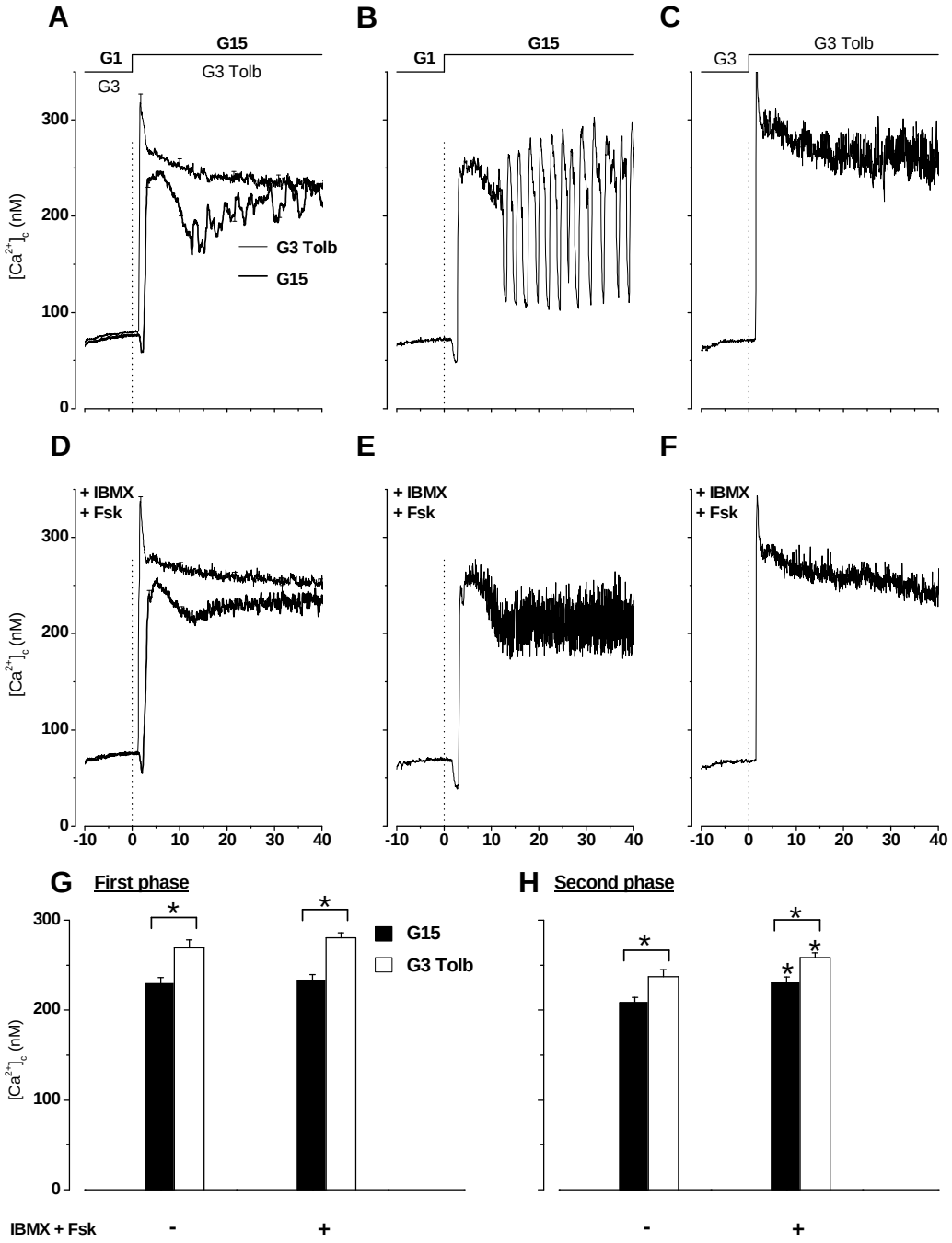

Fig. 27: Effect of cAMP on glucose- and tolbutamide-induced $[Ca^{2+}]_c$ rise in mouse islets. A-F: time course of $[Ca^{2+}]_c$ changes in response to 15 mM glucose (G15) or 500 µM tolbutamide (Tolb) in 3 mM glucose (G3), in the absence (A-C) or presence (D-F) of 10 µM IBMX and 1 µM forskolin (Fsk) throughout. Traces in A and D are mean values for >20 islets. Traces in B, C, E and F are representative of responses in single islets from the same experiment. G and H: $[Ca^{2+}]_c$ integrated over 7 min for first phase (G) (2-9 min for Tolb stimulation and 3-10 min for glucose stimulation) and 30 min for second phase (10-40 min for both). *Significant difference ($P < 0.05$ or less) with controls. Significant differences ($P < 0.01$ or less) between responses to Tolb in G3 and to G15 are shown above pairs of columns (G and H).

tolbutamide stimulation, but the effect is of small amplitude and significant only for the second phase. These results in intact islets therefore support the conclusions, obtained in permeabilized islets or single beta-cells, that the large amplification of insulin secretion by cAMP almost exclusively involves steps parallel or distal to the rise in $[Ca^{2+}]_c$ (Tamagawa et al. 1985, Jones et al. 1986, Renstrom et al. 1997). Increasing cAMP concentrations, however, had a striking qualitative effect on the Ca^{2+} response to glucose: $[Ca^{2+}]_c$ oscillations were abolished, in complete agreement with the abolition of slow waves of membrane potential (Henquin and Meissner 1984). This observation is not incompatible with previous reports on the coordinated glucose-induced oscillations of $[Ca^{2+}]_c$ and cAMP (Dyachok et al. 2008, Idevall-Hagren et al. 2010). The large and sustained increase in cAMP levels induced by the combination of IBMX and forskolin likely perturbs the normal interplay between Ca^{2+} and cAMP signaling, thus abolishing the oscillatory pattern of $[Ca^{2+}]_c$ changes during the second phase of glucose stimulation. It is unclear how this qualitative change quantitatively impacts on insulin secretion.

Studies using TIRFM imaging techniques have identified the granules responsible for the cAMP-dependent increase of exocytotic events to be newcomers (Shibasaki et al. 2007), which appear at the plasma membrane upon stimulation and undergo exocytosis without being docked (Seino et al. 2009). I show here that amplification by cAMP is independent of actin microfilaments. Surprisingly, I found actin depolymerization and stabilization to interfere differently with cAMP-mediated amplification of insulin secretion. Disruption of microfilaments with latrunculin markedly increased the secretory response to glucose and enhanced the amplifying effect of cAMP. When latrunculin was added during glucose + IBMX + forskolin stimulation, its effect was additive to the increase caused by cAMP elevation. On the other hand, microfilament stabilization with jasplakinolide increased the secretory response to glucose to a lesser extent than did latrunculin, but had no effect on the increase caused by cAMP elevation. When jasplakinolide was added during glucose + IBMX + forskolin stimulation, it failed to increase secretion beyond the

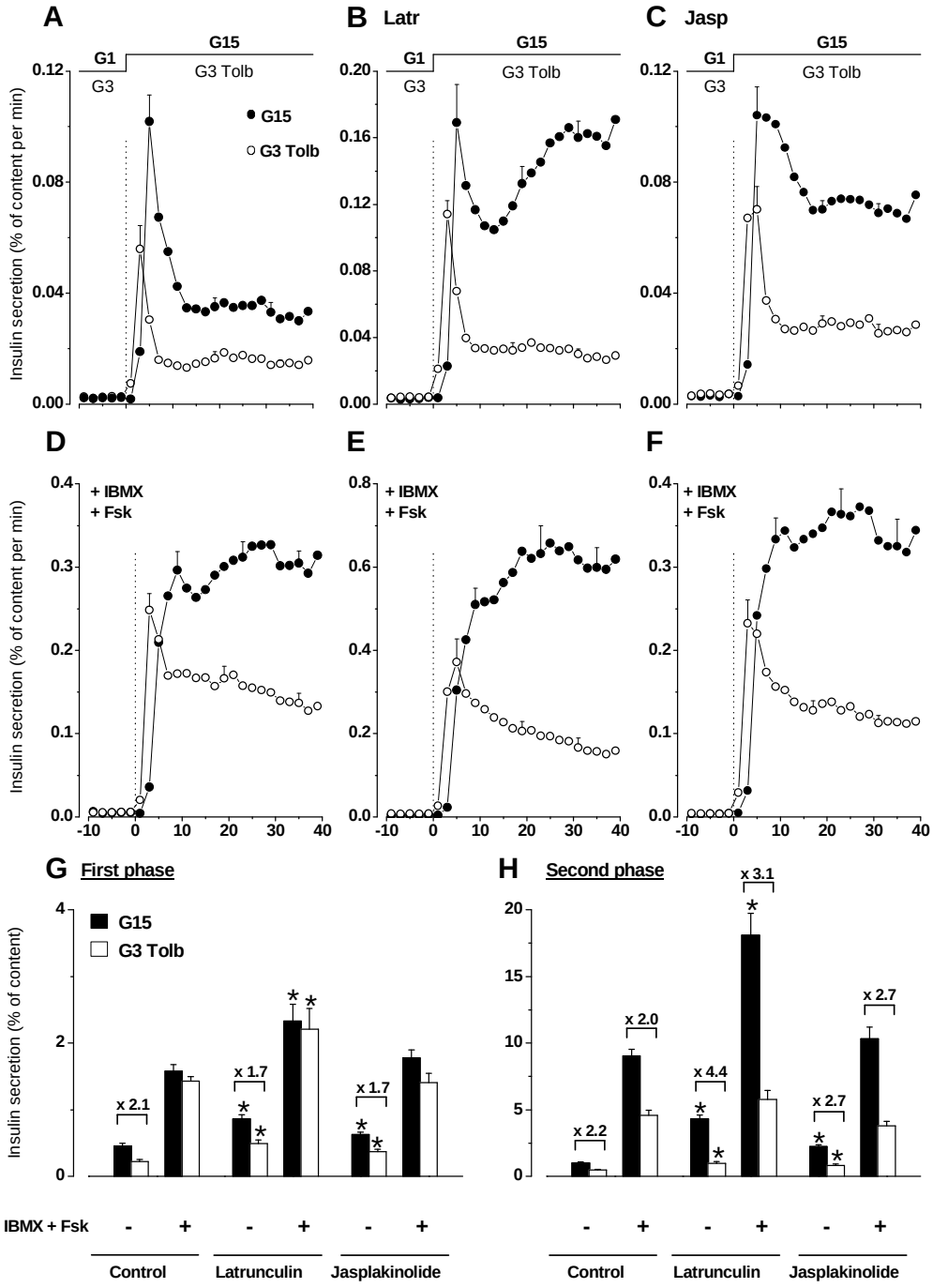

Fig. 28: Effect of cAMP and actin (de)polymerization on glucose- and tolbutamide-induced insulin secretion. A-F: time course of insulin secretion changes in response to 15 mM glucose (G15) or 500 µM tolbutamide (Tolb) in 3 mM glucose (G3), in the absence (A-C) or presence (D-F) of 10 µM IBMX and 1 µM forskolin (Fsk) throughout. Latrunculin (1 µM; Latr) was added to perifusion media in B and E. Islets were treated with jasplakinolide (1 µM; Jasp) for 90 min prior to the experiments in C and F. G and H: insulin secretion integrated over 7 min for first phase (G) (2-9 min for Tolb stimulation and 3-10 min for glucose stimulation) and 30 min for second phase (10-40 min for both). *Significant difference ($P < 0.05$ or less) with appropriate controls (without or with IBMX + Fsk). Significant differences ($P < 0.001$) between responses to Tolb in G3 and to G15 are shown above pairs of columns together with the fold-difference in insulin secretion. Values are means ± SE for 8-12 experiments.

effect of cAMP. These observations indicate that cAMP does not increase secretion by causing actin remodeling but distinguishes between the increases in secretion caused by actin depolymerization and stabilization. Overall our data are in agreement with the proposal that newcomer granules involved in cAMP amplification are located not further than 50-100 nm away from the plasma membrane and that their exocytosis does not require microfilament-dependent movements (Seino et al. 2009).

TIRFM studies have suggested that glucose-induced insulin secretion also involves these newcomer granules (Shibasaki et al 2007). I previously showed and I confirm here that metabolic amplification by glucose is independent of beta-cell microfilaments (Mourad et al. 2010). I also suggested that metabolic amplification might correspond to accelerated priming of highly Ca^{2+}-sensitive granules (Mourad et al. 2010). A question then arises: are metabolic and cAMP amplifications one and the same phenomenon as envisaged by others (Kasai et al. 2010)? Previous studies from the laboratory ruled out the participation of cAMP in metabolic amplification (Gembal et al. 1993, Sato and Henquin 1998). Here the question was addressed by evaluating whether the insulin response to glucose remains larger than that to tolbutamide in low glucose in the presence of saturating cAMP levels. The results show that the difference was unaffected during the second phase of insulin secretion but was suppressed during the first phase, although both phases were amplified by cAMP. We therefore cannot rule out the possibility that cAMP participates in metabolic amplification during the first minutes of glucose stimulation, but it is obvious that the major metabolic amplification taking place during the second phase involves cAMP-independent effects of glucose. These conclusions hold when microfilaments are disrupted or stabilized.

Study 5

Role of actin microfilaments in neurohormonal amplification of insulin secretion: the protein kinase C pathway

Unpublished results

5.1-INTRODUCTION

5.1.1-Insulin secretion and protein kinase C

It has been known for many years that pharmacological activation of protein kinase C (PKC) by phorbol esters increases insulin secretion in a Ca^{2+}-dependent manner (Zawalich et al. 1983). The PKC family comprises three main groups of isoforms: *conventional* PKCs (α, β and γ) that are activated by Ca^{2+} and diacylglycerol (DAG); *novel* PKCs (δ, ε, η and θ) that are activated by DAG only; *atypical* PKCs (ζ, ι/λ and μ) that are independent of Ca^{2+} and DAG but activated downstream of phosphatidylinositol (PI) 3-kinase. Conventional and novel PKCs are activated by phorbol esters (such as PMA). Pancreatic beta-cells express several isoforms of PKC of which PKCα and PKCδ are the most abundant (Onoda et al. 1990), but PKCβ, PKCε, PKCξ are also present (Jones and Persaud 1998, Schmitz-Peiffer and Biden 2008). Stimulation of the PKC pathway occurs via distinct mechanisms and plays distinct roles during receptor-mediated and glucose-mediated insulin secretion.

5.1.2-Acetylcholine (ACh) as an activator of the PLC/PKC pathway

ACh, the main parasympathetic neurotransmitter, binds to M3 muscarinic receptors present in the beta-cell plasma membrane (Henquin et al. 1988, Gautam et al. 2007). These receptors are coupled (via Gq) to a PLC which, upon activation, hydrolyzes membrane phosphoinositides, particularly phosphatidylinositol-4, 5-

bisphosphate (PIP_2) (Yamazaki et al. 2010). In islets as in other cells, this hydrolysis results in the formation of two major products: inositol-1, 4, 5-trisphophate (IP_3) and DAG (Malaisse et al. 1986, Mathias et al. 1985). The two second messengers then activate two distinct pathways that interact to increase the secretory response to glucose. It is indeed important to emphasize from the onset that the effect of ACh on insulin secretion is extremely glucose-dependent. *In vivo*, electrical stimulation of the vagus nerve does not affect plasma insulin during hypoglycemia but increases secretion at higher plasma glucose concentrations (Edwards and Bloom 1986). Similar observations were made when ACh was tested *in vitro* using the whole pancreas (Loubatières-Mariani et al. 1973) or isolated islets (Garcia et al. 1988). This glucose-dependency reflects better efficiency of PKC activation under conditions of elevated $[Ca^{2+}]_c$ as well as greater effects of ACh on membrane potential and $[Ca^{2+}]_c$ in glucose-stimulated beta-cells.

ACh increases beta-cell $[Ca^{2+}]_c$ via two main mechanisms (Gilon and Henquin 2001). IP3 produced from PIP2 binds to its specific receptor (type III in rat, type I in mouse beta cells) located on the endoplasmic reticulum and triggers mobilization of intracellular Ca^{2+} from the ER. Notably, this IP_3-mediated release of ER Ca^{2+} indirectly depends on the ambient glucose concentration because the latter determines the filling state of the ER. The mobilization of intracellular Ca^{2+} by IP3 causes a marked but transient peak of $[Ca^{2+}]_c$ which is followed by an acceleration of glucose-induced $[Ca^{2+}]_c$ oscillations at low ACh concentrations (0.1-1µM) and a sustained $[Ca^{2+}]_c$ plateau at ACh concentrations in the 10-100 µM range (Gilon and Henquin 2001). This increase in beta-cell $[Ca^{2+}]_c$ is caused by membrane depolarization via Na^+ currents and Ca^{2+} entry via VDCC (Rolland et al. 2002) and to a lesser extent by store-operated Ca^{2+} entry (SOCE) (Miura et al. 1997, Liu et al. 1997).

There is no dispute that the concomitant activation of PKC, which can be visualized by the translocation of the enzyme from the cytosol to the plasma membrane (Gromada and Hughes 2006), is an essential component of the increase in

insulin secretion brought about by ACh (Jones and Persaud 1998, Gilon and Henquin 2001). This increase occurs through changes in the sensitivity of the exocytotic process to Ca^{2+} as will be discussed below. Since ACh-dependent activation of beta-cell PKC only occurs when $[Ca^{2+}]_c$ is elevated, it is likely that PKC-dependent effects of ACh on insulin secretion are mainly mediated by the PKCα isoform (Gilon and Henquin 2001).

5.1.3-The PKC pathway in glucose-induced insulin secretion

The role of PKC in nutrient-stimulated insulin secretion remains controversial (Schmitz-Peiffer and Biden, 2008). On one hand, there is evidence that glucose increases DAG in islets via de novo synthesis, but this form of DAG is richer in palmitic acid than arachidonic acid and only poorly activates PKC (Regazzi et al. 1990). Another, indirect, way to activate the enzyme involves anaplerosis-derived malonyl CoA which, by inhibiting CPT1, changes lipid metabolism from β-oxidation toward esterification products (Schmitz-Peiffer and Biden 2008). LCFA-CoAs thus formed during glucose stimulation can activate PKC. This sequence of events has been suggested to contribute to glucose-induced insulin secretion, particularly through the amplifying pathway (Yaney and Corkey 2003), although other effects of LCFA-CoAs have been envisaged (Nolan et al. 2006). On the other hand, several observations cast doubt on the intervention of PKC. Whereas inhibition of conventional PKCs decreases the secretory response to ACh and antagonizes that of phorbol esters, the impact of this inhibition on glucose-induced insulin secretion, if any, is very modest (Zawalich and Zawalich 2001, Carpenter et al. 2004). Although some (not all) studies demonstrated PKC translocation to the plasma membrane in response to glucose, phosphorylation of PKC substrates induced by glucose has been difficult to establish and showed very little correlation with insulin secretion (Carpenter et al. 2004). Activation of atypical PKCs by glucose cannot be excluded (Jones and Persaud 1998).

To complicate the picture, recent studies from the group of Biden rather suggested that PKCε exerts an inhibitory influence on glucose-induced insulin secretion, but this negative effect was only observed under pathological conditions, after islets had been pre-cultured in the presence of fatty-acids. Such a culture is known to impair beta-cell function by poorly defined mechanisms sometimes referred to as "lipotoxicity" (Schmitz-Peiffer et al. 2007, Cantley et al. 2009).

5.1.4-The PKC pathway in the metabolic amplification of insulin secretion

Previous studies from our laboratory have concluded that PKC does not mediate the amplifying effect of glucose when studied under Ca^{2+}-clamped conditions. Thus, glucose amplified the secretory response to 30 mM KCl in islets cultured with PMA to downregulate PKC (Gembal et al. 1993) as well as in islets treated with three different PKC inhibitors (Sato and Henquin 1998). Moreover, exogenous palmitate, which is known to facilitate LCFA-CoA formation in islets, did not mimic the amplifying action of glucose on insulin secretion. (Warnotte et al. 1994). The involvement of PKC in glucose-induced amplification of insulin secretion might also be different between species: the arguments collected in favor have been obtained in rat islets, not in mouse or human islets (Jones et al. 1992, Zawalich and Zawalich 1997, 2001). It has even been proposed that the lack of glucose-induced activation of PKC explains the flat second phase of secretion seen in mice and humans as compared to the ascending second phase seen in rats.

5.1.5-The effectors of PKC on insulin secretion

The mechanism by which PKC activation increases insulin secretion has not yet been fully elucidated. In permeabilized beta-cells, pharmacological activation of PKC increased insulin secretion in the presence of a fixed $[Ca^{2+}]$ (Tamagawa et al. 1985, Regazzi et al. 1989). The resulting conclusion that PKC sensitizes exocytosis to Ca^{2+} stimulation was supported by membrane potential recordings which showed

no effect of PKC activation on beta-cell electrical activity despite markedly increased secretion (Bozem et al. 1987). Studies in single beta-cells also showed that PKC activation by PMA increases depolarization-evoked secretion without affecting Ca^{2+} currents (Ammala et al. 1994). These effects of PKC suggest that its target proteins participate in the final steps of the exocytotic process. Several SNARE proteins possess PKC phosphorylation sites: synaptotagmins, syntaxin 4, VAMP-2 and SNAP-25 (Rutter et al. 2006). Munc18-1 has also been found to be a target for PKCδ: both phosphorylation of the protein and insulin secretion are inhibited in islets from mice with a knock-out of PKCδ (Uchida et al. 2007).

5.1.6-Role of microfilaments in the PKC-mediated increase in insulin secretion

Evidence from other cell types suggests that the regulation of actin dynamics by PKC is a potential mechanism by which the protein kinase modifies granule distribution and their availability for release (Masson-Gadais et al. 1997, Eliyahu et al. 2005, Larsson et al. 2005). The myristoylated-rich C kinase substrate (MARCKS), which binds actin and probably influences vesicle transport, is phosphorylated by PKC in islet cells (Calle et al. 1992). MARCKS activation then leads to activation of other F-actin modifying proteins such as adducin and fascin as well as the Rac and Rho cascades (Masson-Gadais et al. 1997, Larsson et al. 2005). It thus seems plausible that parasympathetic stimulation of beta-cells amplifies glucose-induced secretion by PKC-mediated reorganization of cortical F-actin and/or regulation of some components of the SNARE excocytotic machinery. Indirect evidence on PKC-induced actin remodeling, particularly by the PKCδ isoform (Knutson and Hoenig 1996) as well as Ca^{2+}-dependent association of active PKC with insulin granules (Brocklehurst et al. 1984) suggested that PKC increases insulin secretion through cortical actin reorganization and/or direct action on granules resulting in an increase of the size of the readily releasable pool (Yang and Gillis 2004).

5.2-PERSONAL RESULTS

The aim of my study was two-fold: to investigate whether the polymerization state of beta-cell actin microfilaments is important for PKC amplification of glucose-induced insulin secretion, and to reevaluate the possibility that PKC is involved in metabolic amplification. The experiments were performed with mouse islets treated with latrunculin to depolymerize actin and jasplakinolide to polymerize it. In a first series, PKC activation was achieved indirectly with 1µM ACh. Because changes in $[Ca^{2+}]_c$ also occur in response to ACh, a second series was started using the phorbol ester PMA to activate PKC. These experiments are still underway and the available preliminary results will only be alluded to in the discussion.

5.2.1-Impact of microfilament disruption and stabilization on ACh-mediated amplification of glucose-induced insulin secretion

In a first series of experiments, islets were exposed to ACh during stimulation with 15 mM glucose. In control islets, this caused a ~7-fold increase in secretion that was reversible upon wash-out of the neurotransmitter (Fig. 29). When similar experiments were performed in the presence of 1µM latrunculin, glucose-induced insulin secretion was increased ~4-fold compared to controls as expected (Mourad er al. 2010), and ACh caused a further ~3-fold increase in secretion (Fig. 29). The effects of actin depolymerization and ACh on secretion thus appear to be additive. Disruption of microfilaments does not impair the amplifying action of ACh. A third group of islets was pre-treated with 1µM jasplakinolide to cause irreversible actin polymerization prior to perifusion experiments (Mourad et al. 2010). Actin polymerization increased the secretory response to glucose ~3-fold. ACh still had an amplifying effect but the increase caused by the neurotransmitter did not exceed that seen in control islets (Fig. 29). The effects of actin polymerization and of ACh on secretion are thus not additive.

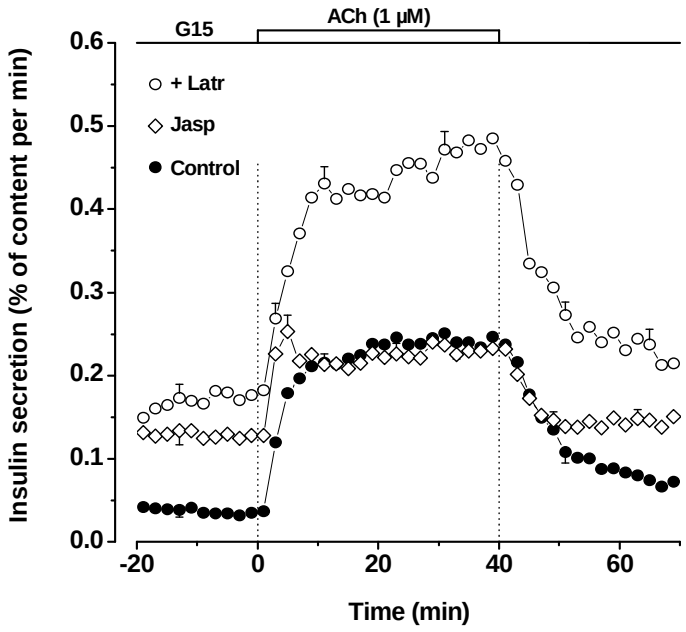

Fig. 29: Effects of actin depolymerization and polymerization on ACh-dependent increase of glucose-induced insulin secretion. Islets were stimulated with 15 mM glucose (G15) at -40 min and acetylcholine (ACh; 1 µM) was added between 0 and 40 min. In one group (o), 1 µM latrunculin (Latr) was present throughout. In another group (◊), the islets were preincubated for 90 min in the presence of 1 µM jasplakinolide (Jasp), but the drug was not added to perifusion media. Values are means ± SE for 9 experiments.

The involvement of actin microfilaments in ACh-mediated amplification of insulin secretion was also investigated using a second protocol. Untreated islets were perifused in 15 mM glucose before being acutely exposed to either latrunculin or jasplakinolide. In agreement with the results shown in study 4, both agents progressively increased glucose-induced insulin secretion, the large effect of latrunculin being reversible in contrast to the smaller one of jasplakinolide (Fig. 30A). When the experiments were repeated in the presence of ACh, the secretory response to 15mM glucose was enhanced ~4-5-fold. Actin depolymerization by latrunculin caused a further 2-fold increase (Fig. 30B) whereas jasplakinolide was ineffective, which confirms non-additivity of the effects of actin polymerization and ACh.

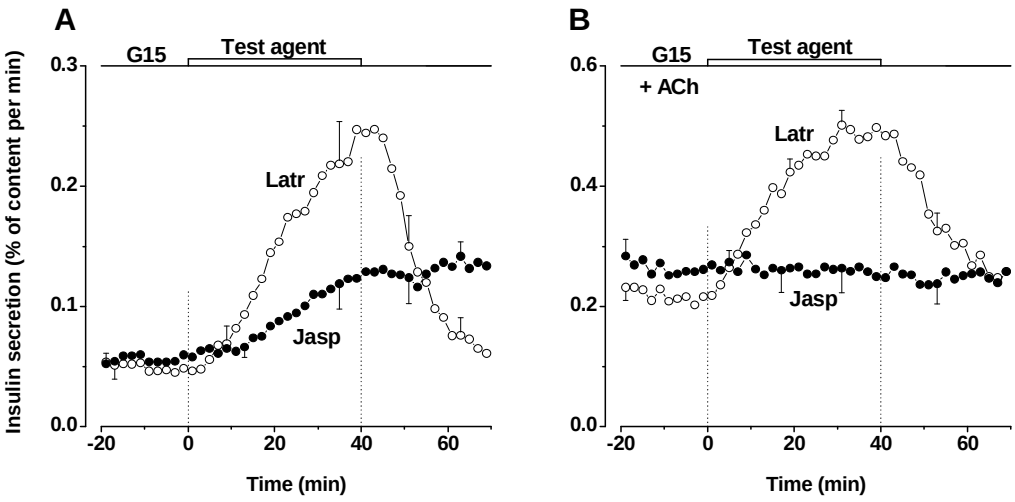

Fig. 30: Effects of actin depolymerization and polymerization on insulin secretion induced by glucose alone or in combination with ACh. A: Mouse islets were stimulated with 15 mM glucose (G15) at -40 min. 1 µM latrunculin (Latr; open circles) or 0.5 µM jasplakinolide (Jasp; filled circles) was added between 0 and 40 min. B: Experiments similar to those shown in A were done in the presence of acetylcholine (ACh; 1 µM) throughout. Note the difference in scale between A and B Values are means ± SE for 5 (Latr) or 4 (Jasp) experiments.

5.2.2-Involvment of PKC in the metabolic amplification of insulin secretion

The experiments from our laboratory which formed the basis for concluding that PKC is not involved in metabolic amplification used the approach of $[Ca^{2+}]_c$ clamping by high KCl in the presence of diazoxide (Gembal et al. 1993, Sato and Henquin 1998). To re-test the hypothesis, I compared $[Ca^{2+}]_c$ and insulin responses in islets stimulated with glucose or tolbutamide, in the presence or absence of ACh.

Figure 31A shows still another series of control islets in which the $[Ca^{2+}]_c$ increase was larger in response to 500µM tolbutamide + 3mM glucose than to 15mM glucose, with a significant difference during first phase only (Fig. 31G and H). ACh slightly increased basal $[Ca^{2+}]_c$ levels, attenuated the initial lowering produced by 15mM glucose, but did not affect the first phase increase in $[Ca^{2+}]_c$ (Fig. 31D, E, G and H). During the second phase, the large oscillations were replaced by small rapid ones (compare the representative traces in Fig 31B and E), which resulted in an

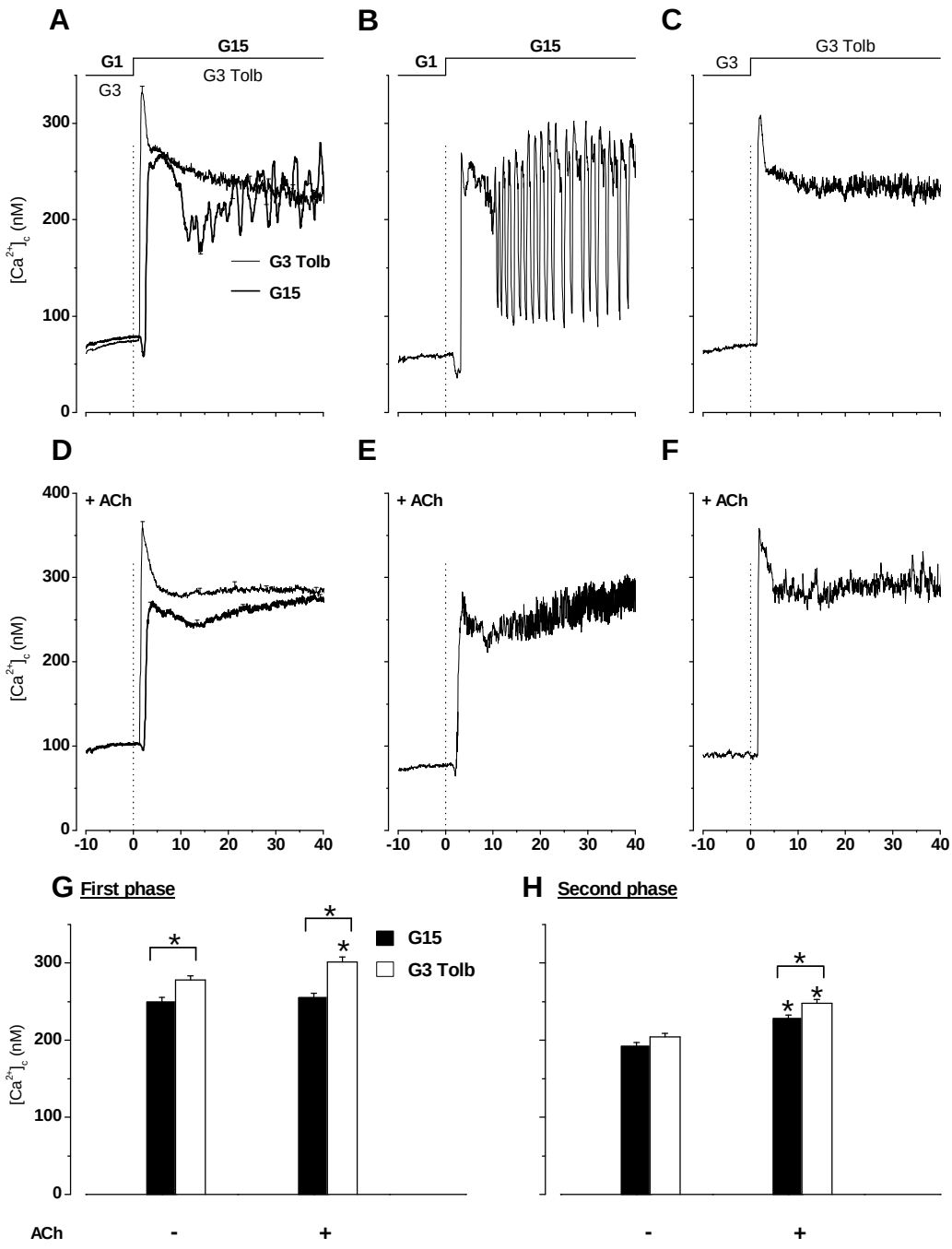

Fig. 31: Effect of ACh on glucose- and tolbutamide-induced $[Ca^{2+}]_c$ rise in mouse islets. A-F: time course of $[Ca^{2+}]_c$ changes in response to 15 mM glucose (G15) or 500 µM tolbutamide (Tolb) in 3 mM glucose (G3), in the absence (A-C) or presence (D-F) of 1 µM acetylcholine (ACh) throughout. Traces in A and D are mean values for >20 islets. Traces in B, C, E and F are representative of responses in single islets from the same experiment. G and H: $[Ca^{2+}]_c$ integrated over 7 min for first phase (G) (2-9 min for Tolb stimulation and 3-10 min for glucose stimulation) and 30 min for second phase (10-40 min for both). *Significant difference ($P < 0.05$ or less) with appropriate controls. Significant differences ($P < 0.001$) between responses to Tolb in G3 and to G15 are shown above pairs of columns (G and H).

increase in average $[Ca^{2+}]_c$ (Fig 31H). The time-course of the response to tolbutamide was not modified by the presence of ACh, but average $[Ca^{2+}]_c$ was increased during both phases (Fig 31G and H). The difference in $[Ca^{2+}]_c$ during first and second phases of glucose and tolbutamide stimulations was not altered by ACh (Fig 31G and H).

Next, we studied insulin secretion under the same experimental conditions (Fig. 32). In controls, the secretory response to glucose was ~2-fold higher than to tolbutamide during both the first and second phases of secretion (Fig. 32A, G, H). Microfilament depolymerization with latrunculin (Fig. 32B) and stabilization with jasplakinolide (Fig. 32C) similarly increased the secretory response to both tolbutamide and glucose, so that the amplifying effect of glucose persisted during first (Fig. 32G) and second (Fig. 32H) phases, as observed in a previous study (Mourad et al. 2010).

As compared with controls (Fig. 32A), ACh increased the secretory response to tolbutamide ~3-fold during first phase and ~2-fold during second phase (Fig 32D, note the differences in scales). The response to glucose was increased ~2-fold during first phase and ~5-fold during the second phase (Fig. 32D, G, H). The amplifying effect of glucose thus persists during both phases of insulin secretion when the PLC/PKC pathway is activated by ACh, but is clearly larger during the second phase. Microfilament depolymerization with latrunculin or stabilization with jasplakinolide did not alter ACh amplification of insulin secretion during stimulation with glucose or tolbutamide (Fig. 32E, G, H). However, as compared with untreated control islets, the amplification by ACh was increased by latrunculin and unchanged by jasplakinolide (Fig. 32F, G and H). Overall the data show that metabolic amplification persists in the presence of ACh, regardless of the polymerization state of microfilaments.

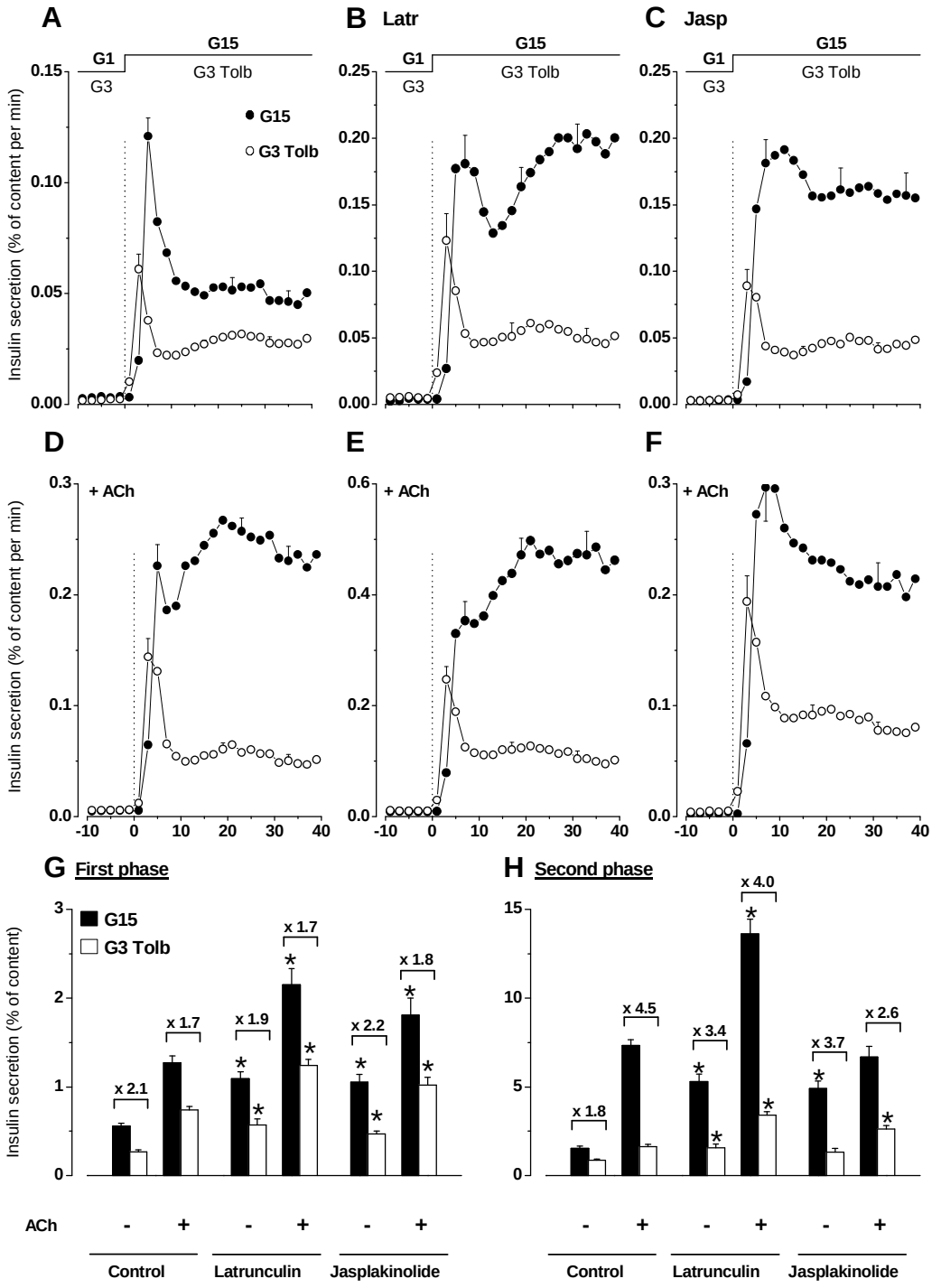

Fig. 32: Effect of ACh and actin (de)polymerization on glucose- and tolbutamide-induced insulin secretion. A-F: time course of insulin secretion changes in response to 15 mM glucose (G15) or 500 µM tolbutamide (Tolb) in 3 mM glucose (G3), in the absence (A-C) or presence (D-F) of 1 µM acetylcholine (ACh) throughout. Latrunculin (1 µM; Latr) was added to perifusion media in B and E. Islets were treated with jasplakinolide (1 µM; Jasp) for 90 min prior to the experiments in C and F. G and H: insulin secretion integrated over 7 min for first phase (G) (2-9 min for Tolb stimulation and 3-10 min for glucose stimulation) and 30 min for second phase (10-40 min for both). *Significant difference ($P < 0.05$ or less) with appropriate controls (with or without ACh). Significant differences ($P < 0.001$) between responses to Tolb in G3 and to G15 are shown above pairs of columns, together with the fold-difference in insulin secretion (G and H). Values are means ± SE for 9-12 experiments.

5.3-DISCUSSION

The results presented here show that actin microfilaments are not required for ACh-induced amplification of insulin secretion. I also show that metabolic amplification of both phases of insulin secretion is independent of PKC activation in mouse islets.

In the experiments presented above, I used ACh to activate PKC via PLC activation. PKC itself has little or no effect on beta-cell electrical activity (Bozem et al. 1987) and $[Ca^{2+}]_c$ (Ammala et al. 1994). In contrast, cholinergic stimulation of beta-cells during glucose stimulation is known to mobilize ER Ca^{2+} stores and increase voltage-dependent Ca^{2+} entry (Gilon and Henquin 2001). Here, I found that ACh increased the Ca^{2+} response to both glucose and tolbutamide. While the time course of $[Ca^{2+}]_c$ elevation in response to tolbutamide was not affected by ACh, the neurotransmitter altered glucose-induced $[Ca^{2+}]_c$ oscillations. This effect can be explained by the changes in beta-cell electrical activity caused by ACh. Thus, 1 µM ACh transforms glucose-induced oscillations of membrane potential into continuous spiking activity, probably due to its Na^+-dependent membrane depolarization and the changes it triggers in voltage-dependent and store-operated Ca^{2+} currents (Gilon and Henquin 2001).

Using latrunculin to depolymerize beta-cell microfilaments, I found the effects of ACh to persist in the absence of functional F-actin. The increase in secretion produced by actin depolymerization was additive with the increase caused by ACh. It thus seems unlikely that PKC-mediated amplification in insulin secretion involves disruption of the cortical actin network. In a striking similarity to what we observed upon PKA activation by cAMP, PKC activation by ACh abolished the increase of glucose-induced secretion by jasplakinolide. While we have no explanation for this phenomenon, it is noteworthy that ACh remained able to amplify the response to glucose after stabilization of actin microfilaments. In fact, insulin secretion rates in response to ACh were similar in control and jasplakinolide-treated islets. It has been suggested that activation of the Rho GTPase Rac1 by protein kinases is important for

secretory granule recruitment by maintaining certain F-actin structures in the cytoplasm (Li et al. 2004). Our observations suggest that F-actin regulation is neither sufficient nor necessary for PKA and PKC activation to amplify glucose-induced insulin secretion.

In voltage-clamped hyperpolarized beta-cells, ACh mobilization of Ca^{2+} from the ER evoked a large increase in membrane capacitance (reflecting exocytosis) which was almost as rapid as that produced by depolarization-induced Ca^{2+} entry (Gromada et al. 1999). This observation prompted the conclusion that secretory granules are in the immediate vicinity of both VDCC and intracellular Ca^{2+} release sites, but it is unclear whether they constitute one or distinct pools. In chromaffin cells, PMA increases the number of docked and readily releasable granules (Gillis et al. 1996, Tsuboi et al. 2001). In beta-cells, PKC activation increases the number of highly Ca^{2+}-sensitive granules (Yang and Gillis 2004). In fact, no study so far has investigated from which pool originates the large number of granules exocytosed during combined glucose and ACh (PKC) stimulation.

My experiments show that insulin secretion in response to G15 + ACh is greater than the response to G3Tolb + ACh during both phases of insulin secretion. Therefore, this difference that reflects the amplifying effect of glucose metabolism cannot be ascribed to activation of the PLC/PKC pathway. The observations that glucose and PKC effects on granular pools are additive (Yang and Gillis 2004) add further support in favor of glucose- and PKC-meditated amplifications being two distinct mechanisms that might however affect the same distal steps in stimulus-secretion coupling. However, we cannot exclude activation by glucose of atypical PKC isoforms (Ca^{2+}- and DAG-independent, and PMA insensitive) different from those activated upon cholinergic stimulation (Jones and Persaud 1998).

One limitation of my series of experiments is that ACh affects $[Ca^{2+}]_c$ in addition to stimulating PKC. This does not invalidate the general conclusions because the changes in $[Ca^{2+}]_c$ were similar for glucose and tolbutamide stimulations. However, experiments using PMA to directly activate PKC in mouse

islets are underway. The results obtained so far indicate persistence of the amplifying effect of glucose when PKC is directly activated by the phorbol ester and thus confirm the interpretation of the results obtained with ACh.

VII. CONCLUSIONS

About 20 years have passed since the identification of a metabolic amplifying pathway in the control of insulin secretion by beta-cells. It is now widely accepted that this metabolic amplification complements the K-ATP channel-dependent production of the triggering Ca^{2+} signal, but there is still no agreement on the mechanisms by which glucose exerts such a crucial effect. The second messengers and effectors involved in this amplification thus remain largely unidentified.

The general view is that metabolic amplification selectively contributes to the second phase of insulin secretion evoked by nutrient secretagogues. Previous work from the laboratory had already cast doubt on this selective contribution. In my thesis studies, performed under conditions where the Ca^{2+} signal was allowed to change freely, I provide evidence that amplifying signals are produced and increase the secretory response already during the first phase and continue to do so during the second phase. This implies that the defects in first phase insulin secretion occurring at the early stages of type 2 diabetes development may have more than one single mechanism.

Other groups have proposed that metabolic amplification is underlain by an acceleration of microfilament- and microtubule-dependent insulin granule recruitment, to make more granules available for the triggering of exocytosis by Ca^{2+}. My results do not support the hypothesis. Thus, metabolic amplification persisted during both phases of insulin secretion after either disruption or stabilization of microfilaments or microtubules. I do not exclude the possibility that glucose has effects on microfilaments and microtubules, as suggested by others, but I show that metabolic amplification is independent of such effects. I even establish that concomitant disruption of microtubules and microfilaments does not perturb the phenomenon. While microfilaments and microtubules may be necessary for long-term replenishment of granular pools, my results show that they are not required for sustaining acute insulin secretion.

It appears therefore that granules affected by glucose-derived amplifying signals do not have to be “recruited” via the cytoskeleton. They are probably located close to the plasma membrane and undergo an accelerated, final, glucose-dependent “priming” step that makes them fully release-competent. From my data I cannot determine whether this acceleration of the priming process affects docked or undocked granules. However, reports using TIRFM to visualize insulin granules beneath the plasma membrane showed that granules released during both phases of the response to glucose are undocked newcomers, and it has been suggested that these newcomers originate from a pool of highly Ca^{2+}-sensitive granules. Because metabolic amplification corresponds to the release of larger numbers of granules for similar or lower $[Ca^{2+}]_c$ in beta-cells, the laboratory previously proposed that it is mediated by an increase in the efficacy of Ca^{2+} on exocytosis. I would rather speculate that metabolic amplification augments the number of release-competent insulin granules in a highly Ca^{2+}-sensitive pool.

I also investigated whether microfilaments are required for amplification of insulin secretion by PKA and PKC, two protein kinases involved in increasing insulin secretion after stimulation by incretin hormones and cholinergic agonists, respectively. The increase in secretion caused by either PKA or PKC was not impaired by microfilament depolymerization and was even additive to that produced by latrunculin. Although PKA or PKC activation still increased the secretory response to glucose after stabilization of microfilaments with jasplakinolide, the effects were not additive for reasons that are not clear. I conclude that PKA and PKC do not increase insulin secretion by causing microfilament-dependent insulin granule recruitment. Like that of glucose, their action may target newcomer granules. In parallel to these studies, I reevaluated the role of the two kinases in metabolic amplification by glucose and found no evidence for the involvement of PKC. Whereas it is clear that cAMP and PKA are not involved in metabolic amplification during the second phase, some doubt persists concerning their contribution to amplification during the first phase.

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Mourad NI, Nenquin M, and Henquin JC. Metabolic amplifying pathway increases both phases of insulin secretion independently of beta-cell actin microfilaments. *Am J Physiol Cell Physiol* 299: C389-398, 2010.

Mourad NI, Nenquin M, and Henquin JC. Metabolic amplification of insulin secretion by glucose is independent of beta-cell microtubules. *Am J Physiol Cell Physiol* 300: C697-706, 2011.

Am J Physiol Cell Physiol 299: C389–C398, 2010.
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Metabolic amplifying pathway increases both phases of insulin secretion independently of β-cell actin microfilaments

Nizar I. Mourad, Myriam Nenquin, and Jean-Claude Henquin

Unit of Endocrinology and Metabolism, University of Louvain Faculty of Medicine, Brussels, Belgium

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Mourad NI, Nenquin M, Henquin J.-C. Metabolic amplifying pathway increases both phases of insulin secretion independently of β-cell actin microfilaments. *Am J Physiol Cell Physiol* 299: C389–C398, 2010. First published May 19, 2010; doi:10.1152/ajpcell.00138.2010.— Two pathways control glucose-induced insulin secretion (IS) by β-cells. The triggering pathway involves ATP-sensitive potassium (K_{ATP}) channel-dependent depolarization, Ca^{2+} influx, and a rise in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$), which triggers exocytosis of insulin granules. The metabolic amplifying pathway augments IS without further increasing $[Ca^{2+}]_c$. The underlying mechanisms are unknown. Here, we tested the hypothesis that amplification implicates actin microfilaments. Mouse islets were treated with latrunculin B and cytochalasin B to depolymerize actin or jasplakinolide to polymerize actin. They were then perifused to measure $[Ca^{2+}]_c$ and IS. Metabolic amplification was studied during imposed steady elevation of $[Ca^{2+}]_c$ by tolbutamide or KCl or by comparing the magnitude of $[Ca^{2+}]_c$ and IS changes produced by glucose and tolbutamide. Both actin polymerization and depolymerization augmented IS triggered by all stimuli without increasing (sometimes decreasing) $[Ca^{2+}]_c$, which indicates a predominantly inhibitory function of microfilaments in exocytosis at a step distal to $[Ca^{2+}]_c$ increase. When $[Ca^{2+}]_c$ was elevated and controlled by KCl or tolbutamide, the amplifying action of glucose was facilitated by actin depolymerization and unaffected by polymerization. Both phases of IS were larger in response to high-glucose than to tolbutamide in low-glucose, although triggering $[Ca^{2+}]_c$ was lower. This difference in IS, due to amplification, persisted when the IS rate was doubled by actin depolymerization or polymerization. In conclusion, metabolic amplification is rapid and influences the first as well as the second phase of IS. It is a late step of stimulus-secretion coupling, which does not require functional actin microfilaments and could correspond to acceleration of the priming process conferring release competence to insulin granules.

biphasic insulin release; cytosolic calcium; exocytosis; insulin granules; pancreatic islets

THE RATE OF INSULIN SECRETION by pancreatic β-cells is controlled by a hierarchical interaction among circulating nutrients, hormones, and neurotransmitters. The preeminent influence of glucose itself is exerted via two signaling pathways that both require metabolism of the sugar in β-cells (1, 11, 39). In the triggering pathway, closure of ATP-sensitive potassium (K_{ATP}) channels by adenine nucleotides permits membrane depolarization, which leads to Ca^{2+} influx through voltage-gated calcium channels and results in an increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) that eventually triggers exocytosis of insulin granules. Simultaneously, glucose activates a metabolic amplifying pathway that does not involve additional action on K_{ATP} channels or further rise in $[Ca^{2+}]_c$ but that augments the secretory response to the triggering Ca^{2+} signal (14). The cellular mechanisms and effectors of this amplification have not yet been identified.

During a hyperglycemic clamp, the increase in plasma insulin concentration is biphasic in humans (4) and rodents (13, 29). In vitro, when a perfused pancreas or perifused isolated islets are challenged by an abrupt and steady increase in the glucose concentration, the acceleration of insulin secretion follows a biphasic pattern with a prominent rapid first phase and a sustained second phase (6, 12, 13, 39, 54). The mechanisms underlying the biphasic kinetics of insulin secretion are incompletely elucidated and probably involve both the time course of intracellular signals and the distribution of insulin granules in distinct pools (12, 27, 30, 35, 41, 50). The first phase is commonly attributed to Ca^{2+}-induced exocytosis of insulin granules from a limited pool of docked (tethered to the plasma membrane) and primed (release competent) granules. In contrast, the second phase is thought to involve functional recruitment or physical translocation of granules to the exocytotic sites. The characteristics and localization of this or these reserve pool(s) are still disputed (3, 16, 19, 31, 35, 38). However, the general view holds that the amplifying action of glucose is necessary for the second phase but not involved in the first phase.

Most actin microfilaments in β-cells are organized in a dense web beneath the plasma membrane (33). Pioneer studies by Lacy et al. (20) and VanObberghen et al. (45) showed that cytochalasins, which depolymerize filamentous actin, facilitate insulin secretion and prompted the suggestion that this filamentous web actually limits access of granules to the exocytotic sites. Extending early models (24, 46), more recent work (9, 47, 50) using single β-cells or insulin-secreting cell lines has proposed that granule translocation along actin filaments or remodeling of the web of actin filaments is involved in the replenishment of releasable granular pools, raising the possibility that these events contribute to the amplifying action of glucose.

The aim of the present study was thus to investigate whether the metabolic amplifying pathway requires functional actin filaments in β-cells. To this end, mouse islets were treated with latrunculin B and cytochalasin B to depolymerize actin or with jasplakinolide to polymerize actin. Three approaches were used to study the effects of glucose during the first and second phases of insulin secretion. $[Ca^{2+}]_c$ was measured in parallel experiments to ascertain that the observed changes in secretion were really due to the amplifying pathway and not to changes in the triggering Ca^{2+} signal.

MATERIALS AND METHODS

The study was approved by, and the experiments were conducted in accordance with, the guidelines of the University of Louvain Animal Research Committee.

Address for reprint requests and other correspondence: J. C. Henquin, Unité d'Endocrinologie et Métabolisme; UCL 55.30, Ave. Hippocrate, 55, B-1200 Brussels, Belgium (e-mail: jean-claude.henquin@uclouvain.be).

Solutions and reagents. The control medium was a bicarbonate-buffered solution containing 120 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 24 mM $NaHCO_3$, 10 mM glucose, and 1 mg/ml BSA. It was gassed with 94 O_2-6 CO_2 to maintain a pH of 7.4. A similar solution was used as test medium in most experiments after adjustment of the glucose concentration and addition of the studied substances. When the concentration of KCl was increased to 30 mM, that of NaCl was decreased accordingly. Most reagents were from Sigma or Merck (Darmstadt, Germany). Diazoxide (a gift from Schering-Plough) and tolbutamide were added from fresh stock solutions in 0.1N NaOH. Cytochalasin B (Sigma), latrunculin B, and jasplakinolide (Calbiochem, San Diego, CA) were added from stock solutions in DMSO, and an equivalent amount of solvent (1 μl/ml) was added to control solutions.

Preparations. Islets were aseptically isolated by collagenase digestion of the pancreas of female C57BL6 mice (8–10 mo) obtained from a local colony. After hand selection, the islets were cultured for about 20 h in RPMI 1640 medium (Invitrogen, Merelbeke, Belgium) kept at 37°C in a 95% air-5% CO_2 atmosphere. The culture medium contained 10 mM glucose, 10% heat-inactivated FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Measurements of insulin secretion. Cultured islets were first preincubated for 90 min at 37°C in 2 ml control medium containing 10 mM glucose and supplemented or not with 10 μM cytochalasin B, 2 μM latrunculin B, or 1 μM jasplakinolide. Two types of techniques were then used to study insulin secretion. In one series, batches of three preincubated islets were incubated in 0.8-ml test medium for 60 min at 37°C. An aliquot of the medium was then taken and diluted before insulin assay. In most experiments, batches of 20 preincubated islets were perifused at 37°C (13) with test solutions, the composition of which was changed as indicated (see Figs. 1–7) . Effluent fractions were collected at 2-min intervals and saved for insulin assay, using rat insulin as a standard (13). At the end of the experiments, the islets were recovered, and their insulin content was determined after extraction in acid-ethanol (26). At the start of perifusions, the islet insulin content was (in ng/islet): controls: 95 ± 3.7, cytochalasin B: 80 ± 3.6, latrunculin B: 83 ± 3.4, and jasplakinolide: 96 ± 4. The slightly lower insulin content ($P = 0.05$) of cytochalasin- or latrunculin-treated islets is attributed to the larger secretion during the preincubation period. Fractional insulin secretion rate was then calculated as the percentage of islet insulin content that was secreted per minute (26). The two phases of insulin secretion were computed over 7 and 20 or 30 min, respectively, as specified (see Figs. 2–7).

Measurements of islet $[Ca^{2+}]_c$ and NAD(P)H. For $[Ca^{2+}]_c$ measurements, cultured islets were loaded with the Ca^{2+} indicator fura-PE3/AM (2 μM) for 2 h at 37°C in 2 ml control medium containing 10 mM glucose and supplemented or not with cytochalasin B, latrunculin B, or jasplakinolide. After loading, the islets were transferred into a chamber mounted on the stage of a microscope and maintained at 37°C. The fura-PE3 probe was excited at 340 and 380 nm, and emission was captured at 510 nm by a Quantem 512QC camera (Roper Scientific, Duluth, GA) and analyzed by the MetaFluor software (Universal Imaging, Downington, PA). Stimulus-induced $[Ca^{2+}]_c$ changes were measured in individual islets and averaged for presentation as mean traces. Average $[Ca^{2+}]_c$ during the first and second phases were computed over the same periods as for insulin secretion. For NAD(P)H measurements, islets were preincubated for 90 min at 37°C in 2 ml control medium without probe. They were then examined with the same system as above and excited at 360 nm with recording of the emitted fluorescence at 470 nm.

Measurements of islet polymerized and depolymerized actin. The ratio of polymerized (filamentous) to depolymerized (globular) actin in control and treated islets was measured using a G-actin/F-actin assay kit (Cytoskeleton, Denver, CO), based on separation of the two fractions by ultracentrifugation and quantification by Western blotting. Batches of 130 islets were treated exactly as for the other experiments and then processed as described by the manufacturer, with one additional step. Proteins in the supernatant fraction were precipitated with the ProteoExtract kit (Calbiochem) because they were too diluted for loading on the gel. The immunoblots were visualized by the SuperSignal West Dura chemiluminescence system (Thermo, Rockford, IL), and the ratio of G-actin to F-actin in each sample was quantified using Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Presentation of results. All experiments have been performed with islets from 3–10 preparations. Results are presented as means ± SE. The statistical significance of differences between means was assessed by ANOVA, followed by a Dunnett's test for comparisons of test groups with controls or a Newman-Keuls test for comparisons between test groups. The unpaired *t*-test was used when only two groups were compared.

RESULTS

Effects of drug treatment on islet actin. To study the role of actin microfilaments in the amplification of insulin secretion by glucose, mouse islets were preincubated for 90 min in the presence of actin-polymerizing or -depolymerizing drugs. We first ascertained their effects on the polymerization state of islet actin. In control islets, 24% of actin was present in filamentous form (Fig. 1). This proportion was decreased to 6 and 10% after treatment with latrunculin B and cytochalasin B, respectively. In contrast, 95% of islet actin was in filamentous form after treatment with jasplakinolide, and this effect was not reversed (92%) by 60 min of washing without drug (Fig. 1).

Impact of actin depolymerization on metabolic amplification of insulin secretion during elevation of islet $[Ca^{2+}]_c$ by KCl. The amplifying action of glucose is classically studied under conditions where $[Ca^{2+}]_c$ is stably elevated by a nonglucose stimulus and minimally affected by glucose itself (11). A first approach consists in holding K_{ATP} channels open with diazoxide and depolarizing islet cells with KCl in the presence of low or high glucose (Fig. 2). The resulting increase in $[Ca^{2+}]_c$ is shown in Fig. 2*A*, *inset*. It was similar in the presence of 1 and 15 mM glucose, during both the first and second phases (Fig. 2*C*). However, insulin secretion was larger in the presence of high glucose (Fig. 2*A*). The difference, which corresponds to

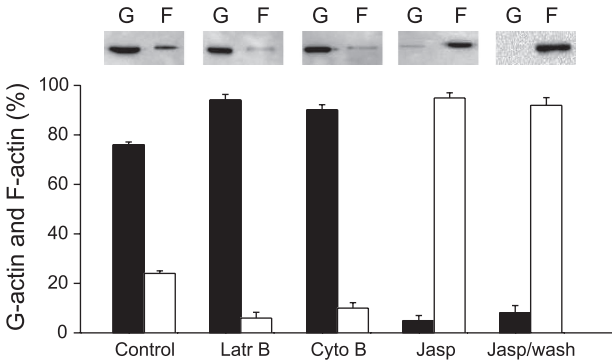

Fig. 1. Proportion of polymerized [filamentous (F)] to depolymerized [globular (G)] actin in control mouse islets and in islets treated with 2 μM latrunculin B (Latr B), 10 μM cytochalasin B (Cyto B), and 1 μM jasplakinolide (Jasp). Islets were incubated for 90 min in control medium containing 10 mM glucose and supplemented or not with the drugs, before being lysed and submitted to ultracentrifugation to separate the 2 actin fractions. Extracts were then subjected to Western blotting for actin. Bars show the percentages of G and F actin in 3–5 experiments, of which 1 representative blot is shown at *top*. In the group Jasp/wash, the islets were treated with jasplakinolide and then washed without drug for 60 min before lysis. The representative blot was not obtained in the same experiment as the other 4. Values are means ± SE.

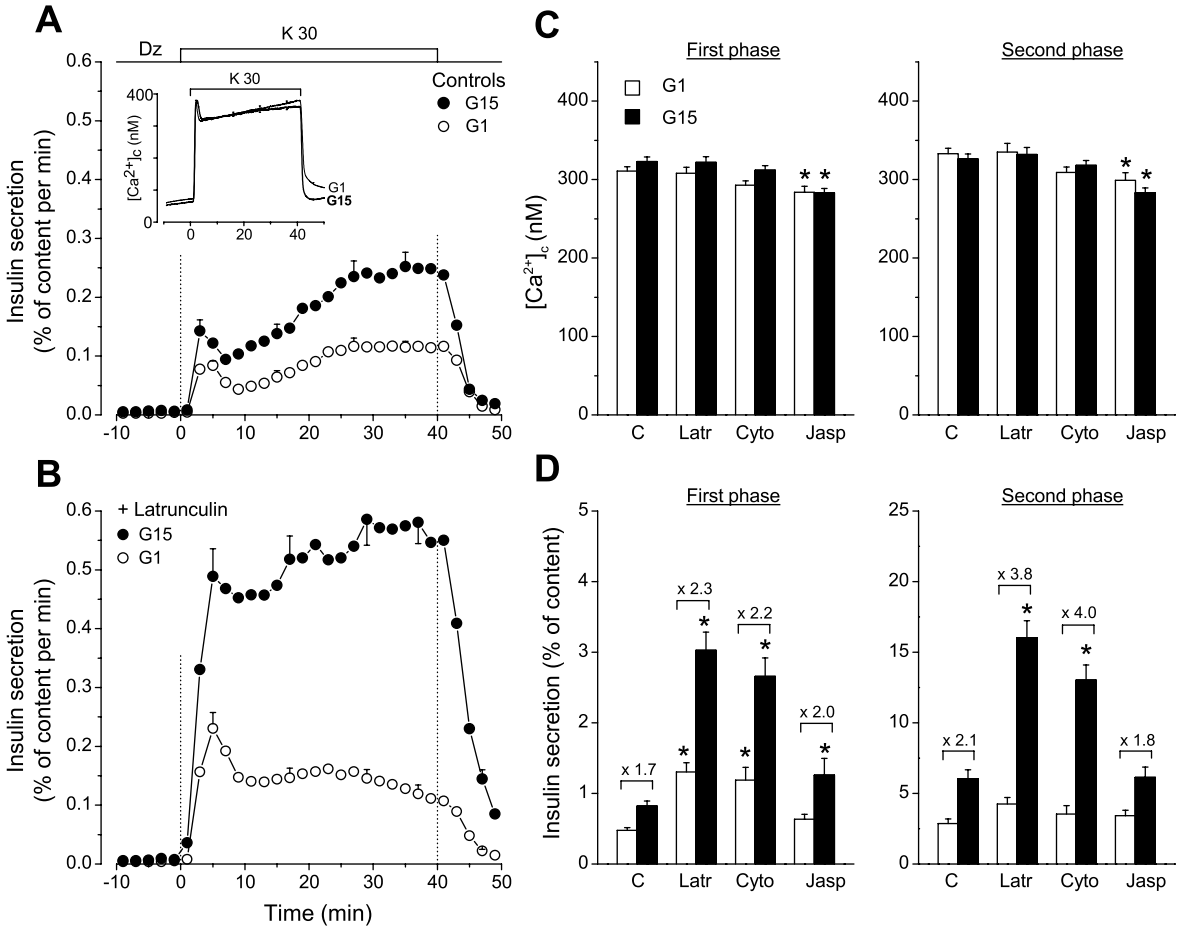

Fig. 2. Effects of actin depolymerization and polymerization on glucose amplification of insulin secretion during clamping of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in islets with KCl. Islets were first preincubated for 90 min with or without 2 μM latrunculin B, 10 μM cytochalasin B, or 1 μM jasplakinolide. They were then used to measure insulin secretion (*A*, *B*, and *D*) or $[Ca^{2+}]_c$ (*C* and in *A*, *inset*). Experiments were performed in the presence of 100 μM diazoxide to prevent any effect of glucose on ATP-sensitive potassium (K_{ATP}) channels and either 1 or 15 mM glucose (G1 or G15). Islets were stimulated by a rise of the KCl concentration from 4.8 to 30 mM (K30) at 0 min. Latrunculin B and cytochalasin B were present during the experiments but jasplakinolide was present during the preincubation period only. *A* and *B*: time course of insulin and $[Ca^{2+}]_c$ changes induced by KCl. *C* and *D*: $[Ca^{2+}]_c$ and insulin responses integrated over 7 min for first phase (2–9 min) and 30 min for second phase (10–40 min). *$P < 0.05$ or less, significant difference with controls (C). Significant differences between responses in G1 and G15 are shown above pairs of columns, together with the fold difference in insulin secretion (*D*). Values are means ± SE for 9 experiments of insulin secretion and 30–40 islets from 5–7 preparations for $[Ca^{2+}]_c$.

the amplification, was significant for both the first phase (1.7-fold) and second phase (2.1-fold) of KCl-induced insulin secretion (Fig. 2*D*).

Similar experiments were then performed after depolymerization of actin microfilaments with latrunculin B or cytochalasin B, which were present during the preincubation period and during $[Ca^{2+}]_c$ and insulin measurements. Latrunculin B (2 μM) augmented KCl-induced insulin secretion in low and high glucose (compare Fig. 2, *A* and *B*) without affecting the increase in $[Ca^{2+}]_c$ (Fig. 2*C*). Notably, only the first phase of KCl-induced insulin secretion was significantly augmented in 1 mM glucose (Fig. 2*D*), whereas the two phases were markedly and significantly larger in the presence of high glucose. As a consequence, the amplifying action of glucose (ratio of the responses in low and high glucose) was increased by latrunculin B, compared with controls (Fig. 2*D*).

Treatment of the islets with cytochalasin B produced similar results. KCl-induced $[Ca^{2+}]_c$ rise was unaffected (Fig. 2*C*), but KCl-induced insulin secretion was augmented, and more so in high than low glucose (Fig. 2*D*), so that the amplifying action of glucose was larger than in control islets. This first series of experiments suggests that depolymerization of actin microfilaments might facilitate metabolic amplification of insulin secretion.

Impact of actin depolymerization on metabolic amplification of insulin secretion during elevation of islet $[Ca^{2+}]_c$ by tolbutamide. A second approach to study the amplifying action of glucose consists in raising the concentration of glucose when all K_{ATP} channels have been closed by a high concentration of sulfonylurea (11). Addition of 500 μM tolbutamide to a medium containing 3 mM glucose markedly increased islet $[Ca^{2+}]_c$ (Fig. 3*A*) and induced insulin secretion (Fig. 3*B*). Subsequently, raising the concentration of glucose to 15 mM caused a rapid drop in $[Ca^{2+}]_c$ followed by a return to levels slightly higher than before glucose stimulation (37) (Fig. 3*A*). Simultaneously with the $[Ca^{2+}]_c$ reascension, insulin secretion increased well above initial levels (Fig. 3*B*). The difference in secretory rate between steady-state periods in tolbutamide + high glucose (40–60 min) and tolbutamide + low glucose (10–30 min) averaged 3.9-fold (Fig. 3*D*) for only a minor,

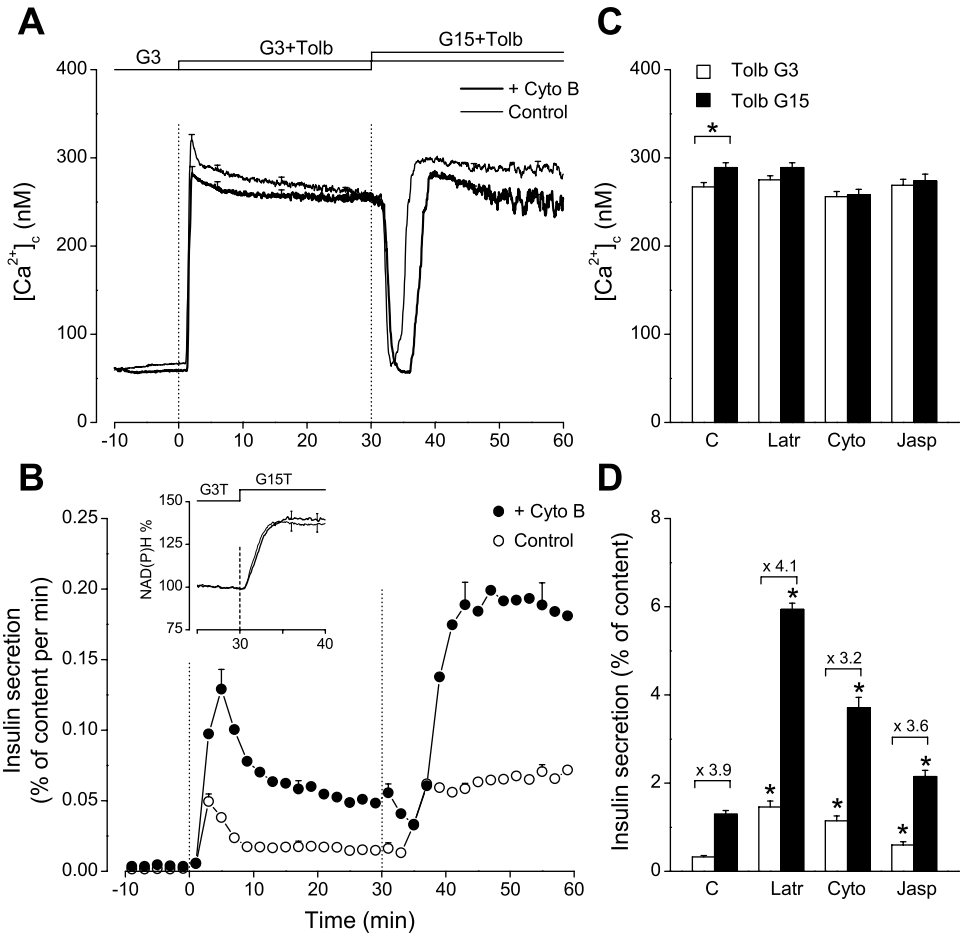

Fig. 3. Effects of actin depolymerization and polymerization on glucose amplification of insulin secretion during islet $[Ca^{2+}]_c$ elevation by tolbutamide. Islets were first preincubated for 90 min with or without 2 μM latrunculin B, 10 μM cytochalasin B, or 1 μM jasplakinolide. They were then used to measure $[Ca^{2+}]_c$ (*A* and *C*) or insulin secretion (*B* and *D*). At 0 min, islets were stimulated with 500 μM tolbutamide (Tolb) in the presence of 3 mM glucose (G3). The concentration of glucose was then raised to 15 mM (G15) at 30 min. Latrunculin B and cytochalasin B were present during the whole experiments, whereas jasplakinolide was present during the preincubation period only. *A* and *B*: time course of $[Ca^{2+}]_c$ and insulin secretion changes in control islets and in islets treated with cytochalasin B. *C* and *D*: $[Ca^{2+}]_c$ and insulin responses integrated over the last 20 min in G3 + Tolb (10–30 min) and in G15 + Tolb (40–60 min). *$P < 0.01$, significant difference with controls. Significant differences ($P < 0.01$) between values in G3 and G15 are shown above pairs of columns, together with the fold change in insulin secretion induced by high glucose (*D*). Values are means ± SE for 8–10 experiments of insulin secretion and 30–38 islets from 5–7 preparations for $[Ca^{2+}]_c$. *B*, *inset*: increase in NAD(P)H fluorescence induced by an increase in the glucose concentration from 3 to 15 mM in islets perifused with 500 μM tolbutamide. Protocol was the same as in the main panel. Control islets are shown by the thin line and islets treated with cytochalasin B by the thick line. Results are expressed as a percentage of the fluorescence in each islet between 25 and 30 min. Values are means ± SE for 12 islets.

although significant, increase in $[Ca^{2+}]_c$ (Fig. 3*C*). It is thus largely attributable to amplification (37).

Cytochalasin B treatment did not affect the $[Ca^{2+}]_c$ rise (Fig. 3*A*) but strongly potentiated insulin secretion (Fig. 3*B*) induced by tolbutamide in 3 mM glucose. When the concentration of glucose was then raised to 15 mM, $[Ca^{2+}]_c$ and insulin secretion transiently decreased before increasing to prestimulatory levels for $[Ca^{2+}]_c$ (Fig. 3, *A* and *C*) and much higher for insulin secretion rate (Fig. 3, *B* and *D*). Similar results were obtained in islets treated with latrunculin B. As a result, the amplifying effect of glucose after actin depolymerization was of a similar relative magnitude (3.2- and 4.1-fold) as in control islets (3.9-fold; Fig. 3*D*). Note, however, that these similar relative changes correspond to larger absolute amounts of insulin (Fig. 3*B*).

The transient decrease in $[Ca^{2+}]_c$ that occurs when the concentration of glucose is increased in the presence of a high concentration of tolbutamide reflects Ca^{2+} uptake by the endoplasmic reticulum and transient β-cell membrane repolarization (8). This decrease in $[Ca^{2+}]_c$ was of longer duration in islets treated with cytochalasin B than in controls (Fig. 3*A*), which delayed the secondary increase in insulin secretion (Fig. 3*B*). Measurements of islet NAD(P)H showed that this delay cannot be attributed to an inhibition or slowing of glucose metabolism by the drug (Fig. 3*B*, *inset*). Other studies (18) have shown that the inhibition of glucose transport and oxidation that cytochalasin B causes in rat islets occurs in non-β-cells. We tentatively suggest that the longer fall in $[Ca^{2+}]_c$, also observed with latrunculin B (not shown), could reflect an influence of actin microfilaments on Ca^{2+} sequestration by the endoplasmic reticulum or on store-operated currents in β-cells as in other cells (21, 25, 36).

Impact of actin depolymerization on metabolic amplification of insulin secretion studied without clamping of islet $[Ca^{2+}]_c$. The amplifying action of glucose can also be studied by comparing the changes in $[Ca^{2+}]_c$ and insulin secretion produced by separate stimulation of islets with either glucose or tolbutamide. As shown in Fig. 4*A*, the increase in $[Ca^{2+}]_c$ produced by 15 mM glucose was delayed compared with that produced by 500 μM tolbutamide in 3 mM glucose, and its magnitude was slightly smaller during both first and second phases (Fig. 4*B*). The onset of insulin secretion was also slower after stimulation with high glucose than tolbutamide (Fig. 4*C*), but the magnitude of the response was larger (~2-fold), during both the first and second phases (Fig. 4*D*).

Islet pretreatment with latrunculin B or cytochalasin B did not significantly affect tolbutamide-induced $[Ca^{2+}]_c$ rise during either phase (Fig. 4*B*), but increased tolbutamide-induced insulin secretion three- to fourfold during both phases (Fig. 4*D*). Both agents also strongly augmented glucose-induced insulin secretion and more so during the second phase (~6-fold) than the first phase (~3-fold; Fig. 4*D*). This increase in secretion occurred while glucose-induced $[Ca^{2+}]_c$ rise was unaffected or slightly attenuated (Fig. 4*B*).

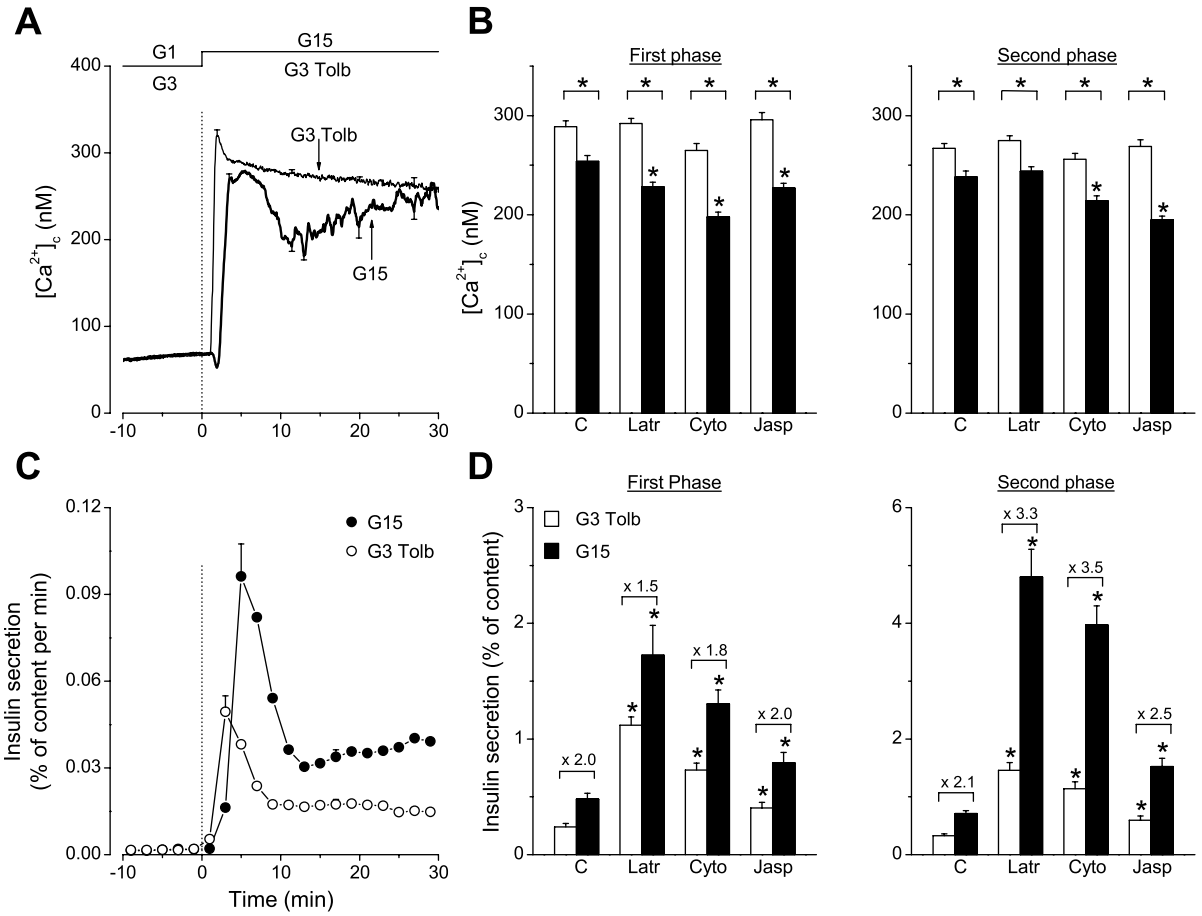

Fig. 4. Comparison of the effects of actin depolymerization or polymerization on glucose- and tolbutamide-induced insulin secretion. Islets were first preincubated for 90 min with or without 2 μM latrunculin B, 10 μM cytochalasin B, or 1 μM jasplakinolide. They were then used to measure $[Ca^{2+}]_c$ (*A* and *B*) or insulin secretion (*C* and *D*). Islets were stimulated with 500 μM tolbutamide (Tolb, thin line or ○) in the presence of 3 mM glucose (G3), or by an increase in the glucose concentration from 1 to 15 mM (G1-G15, thick line or ●). Latrunculin B and cytochalasin B were present during the whole experiments, whereas jasplakinolide was present during the preincubation period only. Experiments with tolbutamide are the same as those of Fig 3. *A* and *C*: time course of $[Ca^{2+}]_c$ and insulin changes in control islets. *B* and *D*: $[Ca^{2+}]_c$ and insulin responses integrated over 7 min for first phase (2–9 min for tobutamide stimulation and 3–10 min for glucose stimulation) and 20 min for second phase (10–30 min for both). $*P < 0.01$, significant difference with controls. Significant differences ($P < 0.01$) between responses to Tolb (open columns) and G15 (filled columns) are shown above pairs of columns, together with the fold difference in insulin secretion (*B* and *D*). Values are means ± SE for 8–10 experiments of insulin secretion and 30–38 islets from 5–7 preparations for $[Ca^{2+}]_c$.

Comparison of the responses of control islets to the two stimuli shows that glucose induced larger first and second phase insulin secretion than tolbutamide in the face of a smaller $[Ca^{2+}]_c$ rise in control islets. The amplifying action of glucose therefore is a rapid and sustained phenomenon. The greater effect of glucose on insulin secretion in spite of a lesser increase in $[Ca^{2+}]_c$ persisted in test islets when the secretion rate had been augmented severalfold by depolymerization of actin microfilaments (Fig. 4*D*). Compared with controls, the difference was even larger during the second phase.

We next investigated whether this amplifying action of glucose during first phase insulin secretion persists when several first phases are triggered consecutively. Control islets were thus stimulated with 15 mM glucose or with 500 μM tolbutamide in low glucose during four periods of 8 min separated by 10-min periods of rest. Each stimulation by glucose induced an increase in $[Ca^{2+}]_c$ that was delayed and smaller (first one) or similar (last three) to those triggered by tolbutamide (Fig. 5*A*). In contrast, insulin secretion was consistently three- to fourfold larger with glucose than tolbutamide (Fig. 5*B*). Similar experiments were then performed with latrunculin B-treated islets. Compared with control islets, the increases in $[Ca^{2+}]_c$ were unaffected, whereas insulin secretion triggered by either glucose or tolbutamide was similarly augmented (Fig. 5*D*; note the difference in scale with Fig. 5*B*). These results show that the amplifying action of glucose is manifest during at least four successive first phases, even after depolymerization of actin microfilaments.

Impact of actin polymerization on metabolic amplification of insulin secretion. Polymerization of actin was achieved by islet treatment with 1 μM jasplakinolide (Fig. 1). However, in contrast to latrunculin B and cytochalasin B, jasplakinolide was present only during the preincubation period because its addition to perifusion solutions was prohibitively expensive. Nonetheless, Fig. 1 shows that actin polymerization by jasplakinolide was not reversible after 1 h in the absence of drug.

Jasplakinolide slightly inhibited the rise in $[Ca^{2+}]_c$ evoked by 30 mM KCl in low or high glucose (Fig. 2*C*) but did not influence KCl-induced insulin secretion except for a slight increase of first phase response in the presence of high glucose (Fig. 2*D*). As a result, glucose amplification of KCl-induced insulin secretion remained unchanged (2.0- vs.

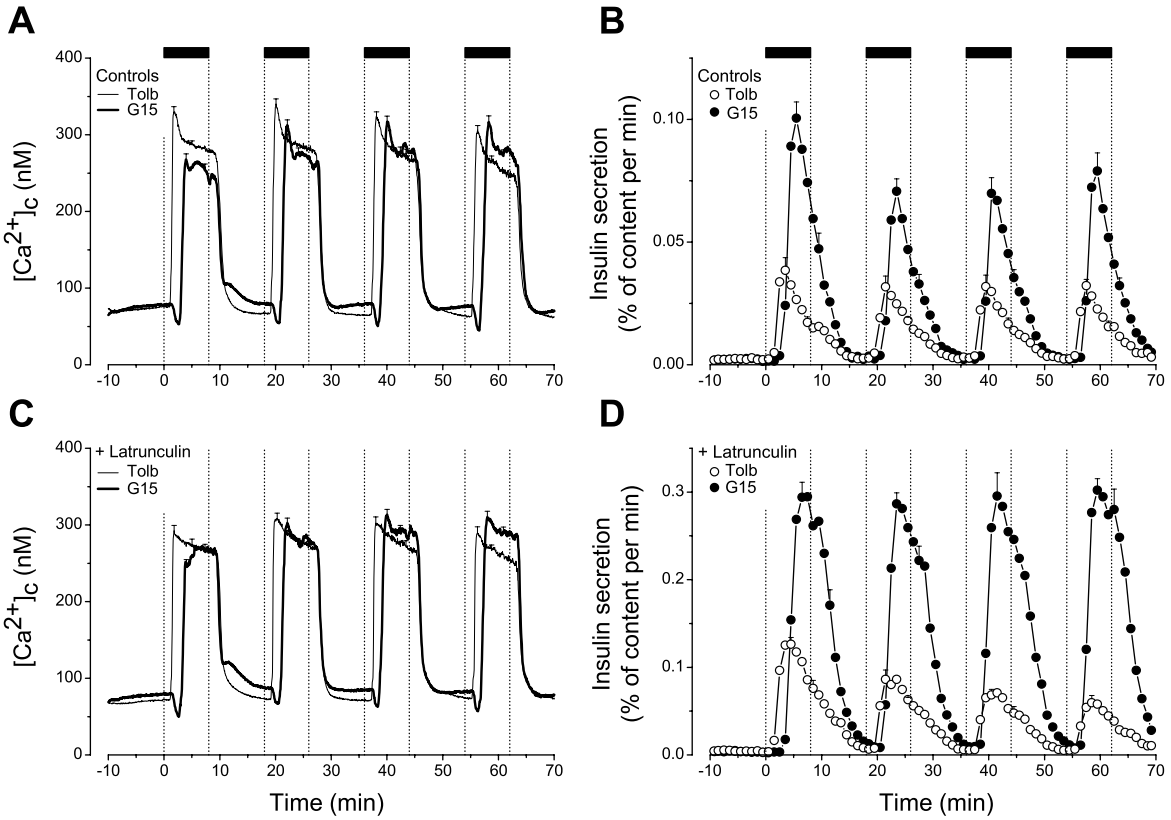

Fig. 5. Amplifying action of glucose during repetitive triggering of first phase of insulin secretion. Islet $[Ca^{2+}]_c$ (*A* and *C*) and insulin secretion (*B* and *D*) were measured in control islets (*A* and *B*) and in islets preincubated (90 min) and perifused with 1 μM latrunculin B (*C* and *D*). Islets were stimulated by addition of 500 μM tolbutamide to 3 mM glucose (G) or by increasing the glucose concentration from 1 to 15 mM. Stimulation was applied 4 times during periods of 8 min (black boxes) separated by 10-min periods of rest. Values are means ± SE for 30–32 islets from 4 preparations for $[Ca^{2+}]_c$ and 5 experiments of insulin secretion.

1.7-fold for first phase and 1.8- vs. 2.0-fold for second phase; Fig. 2*D*).

Pretreatment with jasplakinolide had no effect on tolbutamide-induced $[Ca^{2+}]_c$ rise (Fig. 3*C*) but slightly increased tolbutamide-induced insulin secretion in low glucose. The amplification of secretion produced by high glucose under these conditions was unaffected (3.6-fold in test vs. 3.9-fold in controls; Fig. 3*D*).

Pretreatment with jasplakinolide slightly decreased glucose-evoked $[Ca^{2+}]_c$ rise (Fig. 4*B*) but augmented the two phases of insulin secretion by 1.7- and 2.2-fold (Fig. 4*D*), so that the difference between secretory responses to glucose and tolbutamide was unaltered (Fig. 4*D*). The increase of the two phases of glucose-induced insulin secretion produced by pretreatment of the islets with jasplakinolide is illustrated in Fig. 6, *left* (until 30 min). Jasplakinolide pretreatment also similarly augmented insulin responses to glucose and tolbutamide during successive brief stimulations like those in Fig. 5, so that the difference between effects of the two agents persisted during repetitive first phases (not shown). The amplifying action of glucose on the first phase can thus manifest itself when actin microfilaments are kept polymerized.

Figure 6, *right* (after 30 min), shows that the acceleration of insulin secretion, which followed acute addition of latrunculin B to control islets, did not occur in islets preincubated with jasplakinolide. Although these and above results establish that the effects of a pretreatment with jasplakinolide persist during perifusions subsequently performed in the absence of the drug, a final series of experiments was carried out in its presence. Islets were preincubated with 1 μM jasplakinolide (as in other experiments) and incubated (rather than perifused) also in its presence. As shown in Table 1, jasplakinolide increased insulin

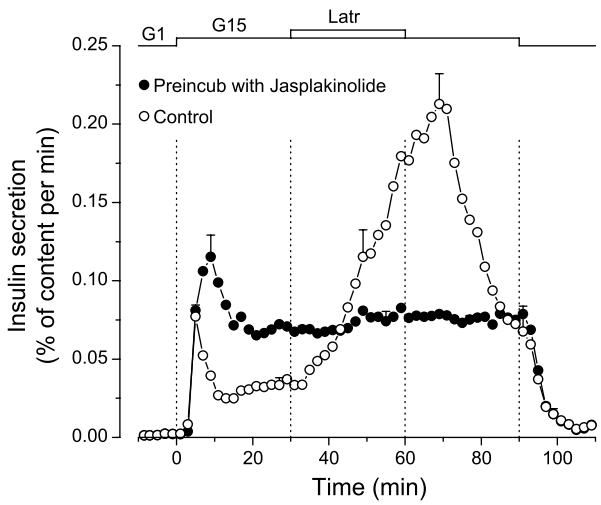

Fig. 6. Preincubation with jasplakinolide prevents the acute effects of latrunculin B on insulin secretion. One group of islets (●) was preincubated for 90 min in the presence of 1 μM jasplakinolide, while the other group (○) was preincubated without drug before being used to study insulin secretion. Latrunculin B (2 μM) was added and the concentration of glucose changed as indicated. Values are means ± SE for 4 experiments.

Table 1. *Effects of Jasplakinolide on insulin secretion by incubated mouse islets*

Incubation Conditions, mM	Insulin Secretion, ng/islet/h	
	Controls	+Jasplakinolide, 1 μM
Experiment 1 (n = 30)		
Glucose 1	0.33 ± 0.05[a]	0.51 ± 0.04[b]
Glucose 15	4.95 ± 0.45[a,c,d]	9.90 ± 0.46[b,c,e]
Experiment 2 (n = 20)		
G3 + Tolbutamide	2.67 ± 0.23[d,f,k]	4.38 ± 0.33[e,g,k]
G15 + Tolbutamide	9.32 ± 0.74[f,h]	13.2 ± 0.88[g,h]
Experiment 3 (n = 25)		
G1 + Diazoxide+KCl	6.35 ± 0.37[i]	7.28 ± 0.73[j]
G15 + Diazoxide+KCl	18.6 ± 1.10[i]	17.7 ± 0.84[j]

Values are means ± SE for n batches of islets studied in 4-6 experiments. Islets were preincubated (90 min) and then incubated (60 min) without or with jasplakinolide. Insulin secretion was measured at the end of the incubation period performed in the presence of the indicated concentration of glucose (G) alone, with 500 μM tolbutamide, or with 100 μM diazoxide + 30 mM KCl. Statistical by ANOVA followed by a Newman-Keuls test: $^{a,b,c,e,f,g,h,i,j}P < 0.001$ and $^{d}P < 0.05$, comparing groups with the same letters. The effect of jasplakinolide in the presence of tolbutamide and low glucose was not significant by ANOVA but reached $^{k}P < 0.0001$ by t-test.

secretion when the islets were stimulated by glucose or tolbutamide but not by KCl. The drug did not alter the amplifying action of 15 mM glucose in the presence of tolbutamide or KCl. The effect of 15 mM glucose alone remained larger than that of tolbutamide in 3 mM glucose when jasplakinolide was present. These results obtained with islets incubated in the presence of jasplakinolide are thus similar to those obtained with islets preincubated with and perifused without jasplakinolide.

Number of insulin granules released during glucose and tolbutamide stimulations. From the measurements of the rate of insulin secretion and the insulin content of the islets, we estimated the number of insulin granules released by each β-cell during the two phases of secretion. These calculations are based on a total of 10,000 granules in control β-cells (7, 32, 41) and 9,000 granules in latrunculin or cytochalasin-treated β-cells (owing to the 10% difference in insulin content of the islets after preincubation with test substance). The results obtained in control and latrunculin-treated islets are shown in Fig. 7. In cytochalasin-treated islets, 120 and 360 granules were released by each β-cell during first and second phases of the response to high glucose, compared with 65 and 100 granules during the two phases of the response to tolbutamide. In jasplakinolide-treated islets, the estimated numbers of secreted granules were 80 and 150 compared with 40 and 60 for each of the two phases of glucose and tolbutamide stimulation, respectively.

DISCUSSION

Our results show that amplification of insulin secretion by glucose is a rapid and sustained phenomenon that influences the magnitude of both first and second phases of the secretory response, without the requirement of functional actin microfilaments in β-cells. We suggest that metabolic amplification is a late event in stimulus-secretion coupling, possibly corresponding to an acceleration of the priming process that confers release competence to insulin granules.

Effects of test agents on actin polymerization. Early electron microscopic studies (45) have shown that cytochalasin B (20 μM) disrupts the web of microfilaments underneath the plasma membrane in rat β-cells. More recent morphological and biochemical approaches have established that latrunculin B (10 μM) decreases actin polymerization (17, 43, 44), whereas jasplakinolide (5 μM) increases actin polymerization (28) in MIN6 insulin-secreting cells. We now show that, in mouse islets, lower concentrations of cytochalasin B (10 μM) and latrunculin B (2 μM) decrease the proportion of polymerized actin to <10%, whereas jasplakinolide (1 μM) causes virtually complete polymerization of actin.

Influence of actin (de)polymerization on insulin secretion. Cytochalasin B has long been known to increase the two phases of insulin secretion induced by high glucose or by a sulfonylurea at nonstimulatory (1.7–5.5 mM) glucose (20, 45). More recently, latrunculin B was found to increase KCl-induced and glucose-induced insulin secretion in MIN6 cells (22, 42, 43). In our study, both cytochalasin B and latrunculin

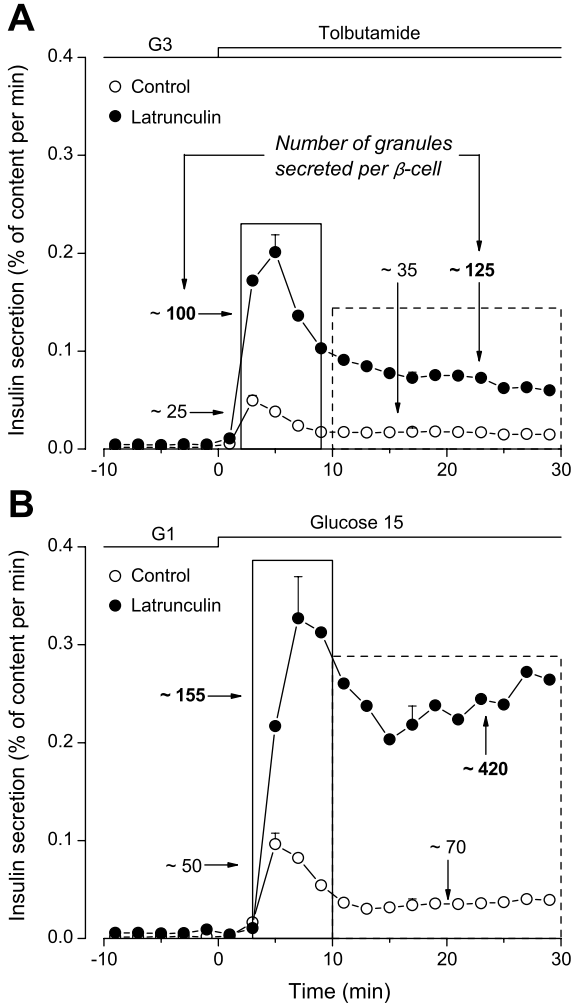

Fig. 7. Estimation of the number of granules released per individual β-cell during the 2 phases of insulin secretion stimulated by tolbutamide (*A*) or glucose (*B*) in control islets and islets treated with latrunculin B. Calculations are based on actual rates of secretion and insulin contents of the islets (see MATERIALS AND METHODS). For facility, we used the numbers of 10,000 granules per β-cell in control islets and 9,000 per β-cell in islets treated with latrunculin B (because the islet content was ~10% lower). First phase was integrated over 7 min (2–9 min for tolbutamide and 3–10 min for glucose), and second phase was integrated over 20 min (10–30 min for both).

B augmented the rapid first phase of insulin secretion induced by high glucose or by tolbutamide and KCl in low glucose. The sustained second phase was also augmented except during stimulation with KCl in low glucose. There is thus agreement that drugs depolymerizing actin filaments in β-cells augment insulin secretion.

In contrast, there is no consensus concerning the impact of actin polymerization by jasplakinolide. The drug was reported to inhibit KCl-induced insulin secretion in RIN cells (51) and MIN6 cells (22) and to block glucose-induced vesicle release in MIN6 cells (44). These inhibitory effects of jasplakinolide in cell lines contrast with the potentiation of glucose-induced insulin secretion observed in incubated mouse islets (28). We confirm the latter observation and further show that jasplakinolide augments both phases of the secretory response to glucose and tolbutamide but does not significantly change the response to KCl. We have no explanation for this selective stimulation that cannot be attributed to distinct effects on $[Ca^{2+}]_c$.

It is thus clear that the peripheral network of actin microfilaments functions as a brake of insulin secretion in primary β-cells (20, 33, 46), and our results show that this brake can be lifted by either polymerization or depolymerization of actin. Other actin microfilaments, localized more deeply in the cytoplasm of β-cells (10), have been suggested to play a positive role in the translocation of insulin granules achieved by motor proteins such as kinesins or myosin 5A (15, 47, 48). However, these experiments have been performed with insulin-secreting cell lines that contain much fewer (<5%) insulin granules than primary β-cells and thus rely more heavily on physical translocation of granules to sustain high insulin secretion rates. This may explain differences in the responses of cell lines and islets to actin depolymerization (23) or polymerization (22, 28, 44, 51).

Influence of actin (de)polymerization on the triggering $[Ca^{2+}]_c$ signal. The possible impact of actin depolymerization on β-cell $[Ca^{2+}]_c$ has been evaluated previously in only one study using HIT cells. Cytochalasin B did not impair the rise in $[Ca^{2+}]_c$ produced by mixed nutrients while paradoxically inhibiting insulin secretion (23). The general idea that the stimulatory effects of the drug on insulin secretion are independent of changes in $[Ca^{2+}]_c$ therefore rests on other approaches. Cytochalasin B was found to increase insulin release induced by a fixed Ca^{2+} concentration in permeabilized rat islets (53), and latrunculin B was found to have a similar effect in MIN6 cells (42). Similar observations were made when exocytosis was evaluated by capacitance recordings in dialyzed INS-1 cells (15). Our experiments in intact islets show that the increase in insulin secretion produced by actin depolymerizing or polymerizing drugs was never accompanied by an increase in $[Ca^{2+}]_c$, regardless of the stimulus, and sometimes even occurred in the face of a small decrease in $[Ca^{2+}]_c$. We can thus safely conclude that actin remodeling affects insulin secretion by mechanisms distal to the rise in $[Ca^{2+}]_c$. Moreover, and most importantly for the present study, the small changes in β-cell $[Ca^{2+}]_c$ that the drugs sometimes produced have no impact on our main question concerning the role of actin filaments in the amplifying action of glucose. Indeed, this action of glucose on insulin secretion was never attributable to a further rise in $[Ca^{2+}]_c$.

Influence of actin (de)polymerization on metabolic amplification. We used three experimental paradigms to study amplification of insulin secretion by glucose and never found it to be impaired by disruption of actin microfilaments. Persistence and even augmentation of amplification after islet treatment with latrunculin B or cytochalasin B do not support the hypothesis that amplification corresponds to glucose-mediated depolymerization of actin. The hypothesis also seems to be ruled out by the preservation of amplification after complete polymerization of actin. It is obvious that metabolic amplification does not require functional actin microfilaments to augment insulin secretion. If granule movements to the exocytotic sites take place during amplification, actin-independent mechanisms must be involved (15, 48, 50). We do not exclude, however, that subtle remodeling of the actin network under the influence of glucose, rather than major shifts in the actin polymerization state, plays a role in stimulus-secretion coupling (50).

Metabolic amplification and pools of insulin granules. Rodent β-cells contain 9,000–13,000 insulin granules (7, 32, 41) distributed into geographically and/or functionally distinct pools. For the sake of facility, we will base our discussion on a total of 10,000 granules. About 50–100 granules are thought to constitute the readily releasable pool, a subset of the ~700 granules that are docked with the plasma membrane. The remaining ~9,000 granules form the reserve pool, of which ~1,500 are near, but not attached to, the plasma membrane (almost docked; Refs. 32, 41). Current models ascribe the rapid first phase of insulin secretion to release of granules from the readily releasable pool, whereas the sustained second phase is thought to require recruitment of granules from a reserve pool (3, 35). It is still debated whether this recruitment involves physical mobilization of granules or a mere change in their properties (priming). The idea that granules must be docked before entering the releasable pool is also challenged (16, 19, 31, 38).

In our control islets, we estimate that ~25 insulin granules were released per β-cell during the first phase (7 min) of tolbutamide-induced insulin secretion and ~35 granules during the second phase (computed over 20 min; Fig. 7*A*). During glucose-induced secretion, while $[Ca^{2+}]_c$ is lower than during tolbutamide stimulation, the numbers are 50 and 70 granules, respectively (Fig. 7*B*). Although these numbers are smaller than the size of the readily releasable pool, the amplifying action of glucose (2-fold) is thus already evident during the first phase. This remains true when the first phase is repetitively triggered by four short pulses of high glucose (Fig. 5*B*). Such observations refute the idea that the first phase is determined only by the amplitude of the triggering Ca^{2+} signal. We (13) previously reached a similar conclusion by comparing the changes in $[Ca^{2+}]_c$ and insulin secretion in response to different glucose concentrations.

Disruption of the web of actin microfilaments by cytochalasin B or latrunculin B increases the number of insulin granules underneath the plasma membrane (17, 45) and, thus, presumably, the size of the readily releasable pool as in chromaffin cells (49). This can explain how ~100 granules can be released during first phase of tolbutamide-induced secretion (Fig. 7*A*). Yet, the amplifying action of glucose on first phase persists after latrunculin treatment (~155 granules) and does not fade out during triggering of several first phases (Fig. 5*D*). Obviously new granules can gain release competence within just a few minutes of glucose stimulation. We therefore propose that the amplifying effect of glucose during the first phase

involves either a rapid action on the readily releasable pool or facilitation of the release of granules that come from another pool, both actions being independent of the operation of actin filaments.

The difference between granule numbers released per β-cell in response to glucose or tolbutamide is even larger during the second phase (Fig. 7). It even becomes impressive during stimulation with KCl in low and high glucose. Thus, in latrunculin-treated islets, high glucose augments the immediate secretory response (7 min) to KCl from 130 to 300 granules and the second sustained response (20 min) from 300 to 1,000 granules per β-cell.

Straub and Sharp (40) have proposed a model in which the releasable pool itself contains a smaller pool of immediately releasable granules (with full competence) and suggested that the rate-limiting step of secretion is the entry into that smaller pool. In their model, amplification corresponds to the conversion of docked granules into immediately releasable granules. Our results are compatible with their proposal that amplification corresponds to acquisition of full release competence by the granules. However, we cannot distinguish between two possibilities: acceleration of the priming process of previously docked granules or facilitation of exocytosis of previously undocked granules. Straub and Sharp (40) also proposed that the size of the first phase is dependent on the size of the immediately releasable pool, which can be influenced by the "history of exposure to glucose," i.e., a time-dependent potentiation mediated by the amplifying pathway. Our interpretation is slightly different. Thus we show that metabolic amplification is a rapid phenomenon that is turned on almost as rapidly as the production of the triggering signal and thereby controls the amplitude of first phase. Our observations also call for amendments to mathematical models of the amplifying pathway (2, 5).

Several recent studies using total internal reflection fluorescence microscopy to visualize exocytotic events indicate that newcomer granules (i.e., granules that belong to neither the readily releasable nor the docked pool) are being released during both the first and second phases (16, 19, 38) or preferentially during the second phase (31) of glucose-induced insulin secretion. An interesting model (34) has proposed that these newcomer granules correspond to a functional pool of highly Ca^{2+}-sensitive granules, which can be increased by glucose (52). In the light of these results and of the current study, we would like to speculate that amplification corresponds to facilitation of release of these newcomer granules.

Conclusions. Amplification of insulin secretion by glucose is rapid and, contrary to current models, influences the first as well as the second phase through actin-independent mechanisms. Because the process corresponds to release of larger numbers of granules for similar or lower $[Ca^{2+}]_c$ in β-cells (11), we previously suggested that it is underlain by an increase in the efficacy of Ca^{2+} on exocytosis. We would now rather suggest that metabolic amplification augments the number of release-competent insulin granules, possibly in a highly Ca^{2+}-sensitive pool.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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Metabolic amplification of insulin secretion by glucose is independent of β-cell microtubules

Nizar I. Mourad, Myriam Nenquin, and Jean-Claude Henquin

Unit of Endocrinology and Metabolism, University of Louvain Faculty of Medicine, Brussels, Belgium

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Mourad NI, Nenquin M, Henquin JC. Metabolic amplification of insulin secretion by glucose is independent of β-cell microtubules. *Am J Physiol Cell Physiol* 300: C697–C706, 2011. First published December 22, 2010; doi:10.1152/ajpcell.00329.2010.—Glucose-induced insulin secretion (IS) by β-cells is controlled by two pathways. The triggering pathway involves ATP-sensitive potassium (K_{ATP}) channel-dependent depolarization, Ca^{2+} influx, and rise in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$), which triggers exocytosis of insulin granules. The metabolic amplifying pathway augments IS without further increasing $[Ca^{2+}]_c$. After exclusion of the contribution of actin microfilaments, we here tested whether amplification implicates microtubule-dependent granule mobilization. Mouse islets were treated with nocodazole or taxol, which completely depolymerized and polymerized tubulin. They were then perifused to measure $[Ca^{2+}]_c$ and IS. Metabolic amplification was studied during imposed steady elevation of $[Ca^{2+}]_c$ by tolbutamide or KCl or by comparing $[Ca^{2+}]_c$ and IS responses to glucose and tolbutamide. Nocodazole did not alter $[Ca^{2+}]_c$ or IS changes induced by the three secretagogues, whereas taxol caused a small inhibition of IS that is partly ascribed to a decrease in $[Ca^{2+}]_c$. When $[Ca^{2+}]_c$ was elevated and controlled by KCl or tolbutamide, the amplifying action of glucose was unaffected by microtubule disruption or stabilization. Both phases of IS were larger in response to glucose than tolbutamide, although triggering $[Ca^{2+}]_c$ was lower. This difference, due to amplification, persisted in nocodazole- or taxol-treated islets, even when IS was augmented fourfold by microfilament disruption with cytochalasin B or latrunculin B. In conclusion, metabolic amplification rapidly augments first and second phases of IS independently of insulin granule translocation along microtubules. We therefore extend our previous proposal that it does not implicate the cytoskeleton but corresponds to acceleration of the priming process conferring release competence to insulin granules.

biphasic insulin release; cytosolic calcium; exocytosis; insulin granules; pancreatic islets

PRECISE INSULIN SECRETION by the endocrine pancreas is essential for normal glucose homeostasis. At the β-cell level, glucose exerts its temporal control and amplitude regulation through a dual mechanism. Glucose metabolism turns on two distinct complementary sequences of events known as the triggering pathway and the metabolic amplifying pathway (reviewed in Ref. 22). The triggering pathway begins with the closure of ATP-sensitive potassium (K_{ATP}) channels, which permits depolarization of the plasma membrane, leading to Ca^{2+} influx via voltage-dependent calcium channels and resulting in an increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) that triggers exocytosis of insulin-containing granules (10, 13, 16, 22). The metabolic amplifying pathway does not directly implicate K_{ATP} channels (1, 21, 53) or any further rise in cytosolic global or subplasmalemmal $[Ca^{2+}]_c$ (21, 46) but augments the secretory response to the triggering Ca^{2+} signal by as yet unresolved mechanisms.

When the concentration of glucose is abruptly and steadily increased, in vitro or in vivo, insulin secretion displays a biphasic time course characterized by a prominent rapid first phase and a sustained second phase (reviewed in Refs. 18, 23, 41, 47, 61). Impairment of the first phase is one of the earliest signs of β-cell dysfunction in type 2 diabetic patients (5, 15). Understanding the cellular mechanisms of this peculiar kinetics has thus aroused much interest but remains a challenging problem for cell physiologists. The picture that emerges from numerous proposed models is that distribution of insulin granules in distinct pools and changes in the triggering Ca^{2+} signal are both involved (4, 13, 18, 23, 41, 47, 53, 60). The first phase is ascribed to Ca^{2+}-induced exocytosis of a small pool of readily releasable insulin granules. The second phase is thought to require functional recruitment and physical mobilization of reserve granules to replenish the releasable pool near exocytotic sites. Several laboratories have suggested that metabolic amplification only contributes to second phase and corresponds to acceleration of the recruitment process through actions on the cytoskeleton of microfilaments and microtubules (4, 13, 47, 58, 60).

Pharmacological disruption of the web of actin microfilaments beneath the plasma membrane of β-cells facilitates insulin secretion (57), and remodeling of this web might play a role in the secretory response to glucose (60). However, we recently reported that metabolic amplification influences the first as well as the second phase independently of functional actin microfilaments (39).

The proposition that β-cell microtubules are active players in insulin secretion originates from experiments by Lacy et al. (33) and Malaisse et al. (8, 34, 51), who showed that drugs known to interfere with tubulin polymerization (vinblastine, vincristine, and colchicine) generally inhibited insulin secretion from pieces of rat pancreas, isolated islets, or the perfused pancreas. The changes in secretion were, however, variable, dependent on the duration of drug application and the type of stimulus (56), and sometimes small (38) or even absent (17). Nonetheless, the bulk of results supported models in which the second phase of glucose-induced insulin secretion is dependent on mobilization of secretory granules along microtubules (26, 35). This interpretation was supported by cinematographic evidence that vinblastine and colchicine inhibited glucose-induced granular movements in β-cells (30, 32, 50). More recently, conventional kinesin, which provides ATP-dependent motor activity to transport secretory vesicles along microtubules, has been identified in insulin-secreting cells lines and in islets (2, 37). It was then proposed that one consequence of the rise in β-cell $[Ca^{2+}]_c$ produced by glucose is a dephosphorylation of kinesin, with increase of its motor activity and

Address for reprint requests and other correspondence: J. C. Henquin, Unité d'Endocrinologie et Métabolisme; UCL 55.30, Ave. Hippocrate, 55, B-1200 Brussels, Belgium (e-mail: jean-claude.henquin@uclouvain.be).

acceleration of insulin granule movements along microtubules (9). This scenario was compatible with observations that inactivation of kinesin by antisense nucleotides or dominant-negative mutants inhibits insulin granule movement and insulin secretion (37, 58, 59). Glucose-induced granular movement along microtubules is thus a plausible mechanism of metabolic amplification.

The aim of the present study therefore was to investigate whether the metabolic amplifying pathway requires functional microtubules in β-cells. To this end, mouse islets were treated with nocodazole to depolymerize tubulin and disrupt microtubules or treated with taxol to polymerize tubulin and stabilize microtubules (28, 29). Insulin secretion and islet $[Ca^{2+}]_c$ were measured in dynamic perifusion systems to distinguish between the two phases of secretion and ascertain that the observed changes in secretion were really due to the amplifying pathway and not to alterations of the triggering Ca^{2+} signal.

MATERIALS AND METHODS

The study was approved by, and the experiments were conducted in accordance with, the guidelines of the University of Louvain Animal Research Committee.

Solutions and reagents. The control medium was a bicarbonate-buffered solution containing 120 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 24 mM $NaHCO_3$, 10 mM glucose, and 1 mg/ml bovine serum albumin. The solution was gassed with 94% O_2-6% CO_2 to maintain a pH of 7.4. A similar solution was used as test medium in most experiments after adjustment of the glucose concentration and addition of the studied substances. When the concentration of KCl was increased to 30 mM, that of NaCl was decreased accordingly. Most reagents were from Sigma or Merck (Darmstadt, Germany). Diazoxide (a gift from Schering-Plough, Brussels) and tolbutamide were added from fresh stock solutions in 0.1 N NaOH. Nocodazole, taxol, cytochalasin B (Sigma), and latrunculin B (Calbiochem, San Diego, CA) were added from stock solutions in DMSO, and an equivalent amount of solvent (1 μl/ml) was added to control solutions. A few experiments were also performed with vinblastine (Calbiochem) added from a stock solution in water.

Preparations. Islets were aseptically isolated by collagenase digestion of the pancreas of female C57BL6 mice (8–10 mo) obtained from a local colony. After hand selection, the islets were cultured for about 20 h in RPMI 1640 medium (Invitrogen, Merelbeke, Belgium) kept at 37°C in a 95% air-5% CO_2 atmosphere. The culture medium contained 10 mM glucose, 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Measurements of insulin secretion. Cultured islets were first preincubated for 90 min at 37°C in 2 ml control medium containing 10 mM glucose and supplemented or not with 10 μM nocodazole or 5 μM taxol. Batches of 20 preincubated islets were then perifused at 37°C (24) with test solutions, the composition of which was changed as indicated at the top of the figures. Effluent fractions were collected at 2-min intervals and saved for insulin assay, using rat insulin as a standard (24). At the end of the experiments, the islets were recovered, and their insulin content was determined after extraction in acid-ethanol (40). At the start of perifusions, the islet insulin content was (ng per islet) the following: controls, 102 ± 4.1; nocodazole, 98 ± 4.3; and taxol, 103 ± 5.7. Fractional insulin secretion rate was then calculated as the percentage of islet insulin content that was secreted per minute (40). The two phases of insulin secretion were computed over 7 and 20 or 30 min, respectively, as specified in the legend of the appropriate figures.

Measurements of islet $[Ca^{2+}]_c$. Cultured islets were loaded with the Ca^{2+} indicator fura-PE3/AM (2 μM) for 2 h at 37°C, in 2 ml control medium containing 10 mM glucose, and supplemented or not with nocodazole, vinblastine, or taxol. After loading was completed, the islets were transferred into a chamber mounted on the stage of a microscope and maintained at 37°C. The fura-PE3 probe was excited at 340 and 380 nm, and emission was captured at 510 nm by a Quantem 512QC camera (Roper Scientific, Duluth, GA) and analyzed by the MetaFluor software (Universal Imaging, Downington, PA). Stimulus-induced $[Ca^{2+}]_c$ changes were measured in individual islets and averaged for presentation as mean traces. Average $[Ca^{2+}]_c$ during first and second phases were computed over the same periods as for insulin secretion.

Measurements of islet polymerized and depolymerized tubulin. The ratio of polymerized tubulin (microtubules) to unpolymerized (free) tubulin in control and treated islets was measured using a microtubules/tubulin assay kit (Cytoskeleton, Denver, CO), based on separation of the two fractions by ultracentrifugation and quantification by Western blot analysis. Batches of 130 islets were treated exactly as for the other experiments and then processed as described by the manufacturer with one additional step. Proteins in the supernatant fraction were precipitated with the ProteoExtract kit (Calbiochem, San Diego, CA) because they were too diluted for loading on the gel. The immunoblots were visualized by the SuperSignal West Dura chemiluminescence system (Thermo, Rockford, IL), and the ratio of microtubles to tubulin in each sample was quantified using the Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Measurements of islet polymerized and depolymerized actin. These experiments were performed to assess possible effects of taxol and nocodazole on islet microfilaments. The ratio of polymerized (filamentous) to depolymerized (globular) actin was measured exactly as in our previous study (39).

Presentation of results. All experiments have been performed with islets from 3 to 10 preparations. Results are presented as means ± SE or SD as indicated. The statistical significance of differences between means was assessed by ANOVA, followed by a Dunnett's test for comparisons of test groups with controls, or a Newman-Keuls test for comparisons between test groups.

RESULTS

Effects of drug treatment on islet tubulin. The role of β-cell microtubules in metabolic amplification of insulin secretion was studied by treating mouse islets with tubulin-polymerizing or -depolymerizing agents (28, 29). We first ascertained their effects on the polymerization state of islet tubulin. In control islets, 34% of tubulin was present in the form of microtubules (polymerized) and 66% in free (unpolymerized) form (Fig. 1). This proportion is similar to that previously found in rat islets (36, 44). After 90 min of preincubation with vinblastine or nocodazole (two microtubule disrupters), islet tubulin was almost completely depolymerized. Preincubation with taxol, a microtubule stabilizer, produced the opposite effect, causing virtually complete polymerization of islet tubulin (Fig. 1, *left*). These biochemical results in islets agree with the morphological disappearance and stabilization of microtubules in insulin-secreting HIT-cells treated with nocodazole and taxol, respectively (14). Figure 1 (*right*) also shows that latrunculin B and cytochalasin B, two drugs causing depolymerization of actin microfilaments (39), did not influence tubulin polymerization and did not interfere with the decrease and increase in tubulin polymerization produced by nocodazole and taxol, respectively.

Inhibition of islet $[Ca^{2+}]_c$ by vinblastine. Islet treatment with 10 μM vinblastine inhibited glucose-induced insulin secretion by about 30%, as previously reported (33), but unexpectedly caused a similar decrease in $[Ca^{2+}]_c$. Vinblastine also inhibited KCl-induced $[Ca^{2+}]_c$ rise by 25–30%. Because these changes in $[Ca^{2+}]_c$ complicate the interpretation of secretion

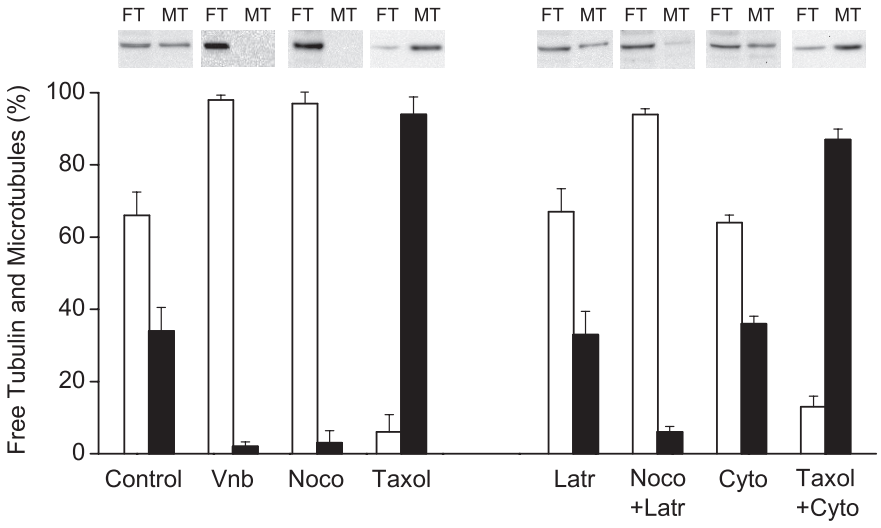

Fig. 1. Proportion of unpolymerized tubulin [free tubulin (FT)] to polymerized tubulin [microtubular (MT)] in mouse islets. *Left*: control islets and islets treated with 10 μM vinblastine (Vnb), 10 μM nocodazole (Noco), or 5 μM taxol. *Right*: islets treated with 1 μM latrunculin B (Latr) or 10 μM cytochalasin B (Cyto) alone or combined with Noco or taxol. Islets were incubated for 90 min in control medium containing 10 mM glucose and supplemented or not with the drugs. They were then lysed and submitted to ultracentrifugation to separate the two tubulin fractions. Extracts were later subjected to Western blot analysis for tubulin. Bars show the percentages of FT and MT in 6 control and 3–5 test experiments, of which one representative blot is shown at *top*. Not all blots were obtained in the same experiment. Values are means ± SD.

changes, the drug was not further used, and microtubules were depolymerized by nocodazole (Fig. 1) that had little effect on $[Ca^{2+}]_c$.

Impact of microtubule disruption and stabilization on metabolic amplification of insulin secretion during elevation of islet $[Ca^{2+}]_c$ by KCl. Amplification of insulin secretion by glucose is classically studied under conditions where the triggering $[Ca^{2+}]_c$ signal is stably elevated by a nonglucose stimulus and minimally affected by glucose itself (21). While K_{ATP} channels were held open with diazoxide, islet cells were depolarized with KCl in the presence of low or high glucose (Fig. 2). The resulting increase in $[Ca^{2+}]_c$ was similar in the presence of 1 and 15 mM glucose (Fig. 2*B*), but insulin secretion was larger in the presence of high glucose (Fig. 2*A*). The approximately twofold difference in secretion (Fig. 2*F*) for a similar $[Ca^{2+}]_c$ (Fig. 2*C*) corresponds to the metabolic amplification.

Similar experiments were then performed after depolymerization of tubulin with 10 μM nocodazole that was present during the preincubation period and during $[Ca^{2+}]_c$ and insulin measurements (Fig. 2*D*). Nocodazole slightly (15%, $P < 0.05$) attenuated KCl-induced $[Ca^{2+}]_c$ rise in 1 mM glucose but not in 15 mM glucose (Fig. 2*C*). When compared with controls, insulin secretion was not significantly affected, so that the amplifying action of high glucose (2.1-fold) was unaltered (Fig. 2, *D* and *F*).

We then tested the effects of 5 μM taxol, a microtubule-stabilizer (Fig. 2*E*). The time course of KCl-induced $[Ca^{2+}]_c$ increase was unaffected (Fig. 2*E*, *inset*), but its magnitude was reduced by ~20% in low and high glucose (Fig. 2*C*). This may contribute to the ~50% inhibition of KCl-induced insulin secretion that taxol produced at both glucose concentrations (Fig. 2*F*). Importantly, the secretory response induced by a similar $[Ca^{2+}]_c$ (Fig. 2*C*) was doubled by high glucose as in controls (Fig. 2*F*). Metabolic amplification was thus unaltered by microtubule disruption or stabilization under these conditions of depolarization with KCl.

Impact of microtubule disruption and stabilization on metabolic amplification of insulin secretion during elevation of islet $[Ca^{2+}]_c$ by tolbutamide. The amplifying action of glucose can also be studied during forced closure of all K_{ATP} channels with a high concentration of sulfonylurea (21). The experiments illustrated in Fig. 3 (*A* and *B*) start 10 min after addition of 500 μM tolbutamide to a medium containing 3 mM glucose. In control islets, $[Ca^{2+}]_c$ was markedly elevated and insulin secretion was stimulated (Figure 3, compare with dotted lines labeled G3 and showing control data in the absence of tolbutamide). Subsequently raising the concentration of glucose to 15 mM caused a rapid but transient drop in $[Ca^{2+}]_c$, which reflects Ca^{2+} uptake by the endoplasmic reticulum and transient β-cell membrane repolarization (11, 12). Concomitantly, with the reascension of $[Ca^{2+}]_c$ to levels slightly higher than before glucose stimulation (48), insulin secretion increased markedly (Fig. 3*B*). The difference in $[Ca^{2+}]_c$ between steady-state periods in tolbutamide + high glucose (10–30 min) and tolbutamide + low glucose (−20–0 min) was small, though significant (Fig. 3*C*), whereas the difference in secretory rate averaged 4.2-fold (Fig. 3*D*) and is thus largely attributable to amplification (48).

Islet treatment with nocodazole did not affect $[Ca^{2+}]_c$ or insulin secretion in the presence of tolbutamide and 3 or 15 mM glucose (Fig. 3). The amplification of secretion (4.3-fold difference between responses in low and high glucose) was thus similar to that in control islets (Fig. 3*D*). Islet treatment with taxol slightly lowered $[Ca^{2+}]_c$ (Fig. 3, *A* and *C*) and similarly inhibited (40%) insulin secretion (Fig. 3, *B* and *D*) in the presence of tolbutamide and low or high glucose. The amplifying action of glucose on secretion therefore remained unaltered (4.2-fold) (Fig. 3*D*).

Impact of microtubule disruption and stabilization on metabolic amplification of insulin secretion studied without clamping of islet $[Ca^{2+}]_c$. The amplifying action of glucose was next studied by comparing the changes in $[Ca^{2+}]_c$ and insulin secretion produced by separate stimulation of islets with either 15 mM glucose or 500 μM tolbutamide in 3 mM glucose (Fig. 4). In control islets, tolbutamide produced a rapid increase in $[Ca^{2+}]_c$ characterized by an initial peak followed by a steady but slowly declining elevation (Fig. 4*A*, *trace C*) and triggered biphasic insulin secretion (Fig. 4*D*, filled circles). Glucose also produced a biphasic increase in $[Ca^{2+}]_c$ that was slightly delayed when compared with the response to tolbutamide (Fig. 4*B*, *trace C*), because of a small initial

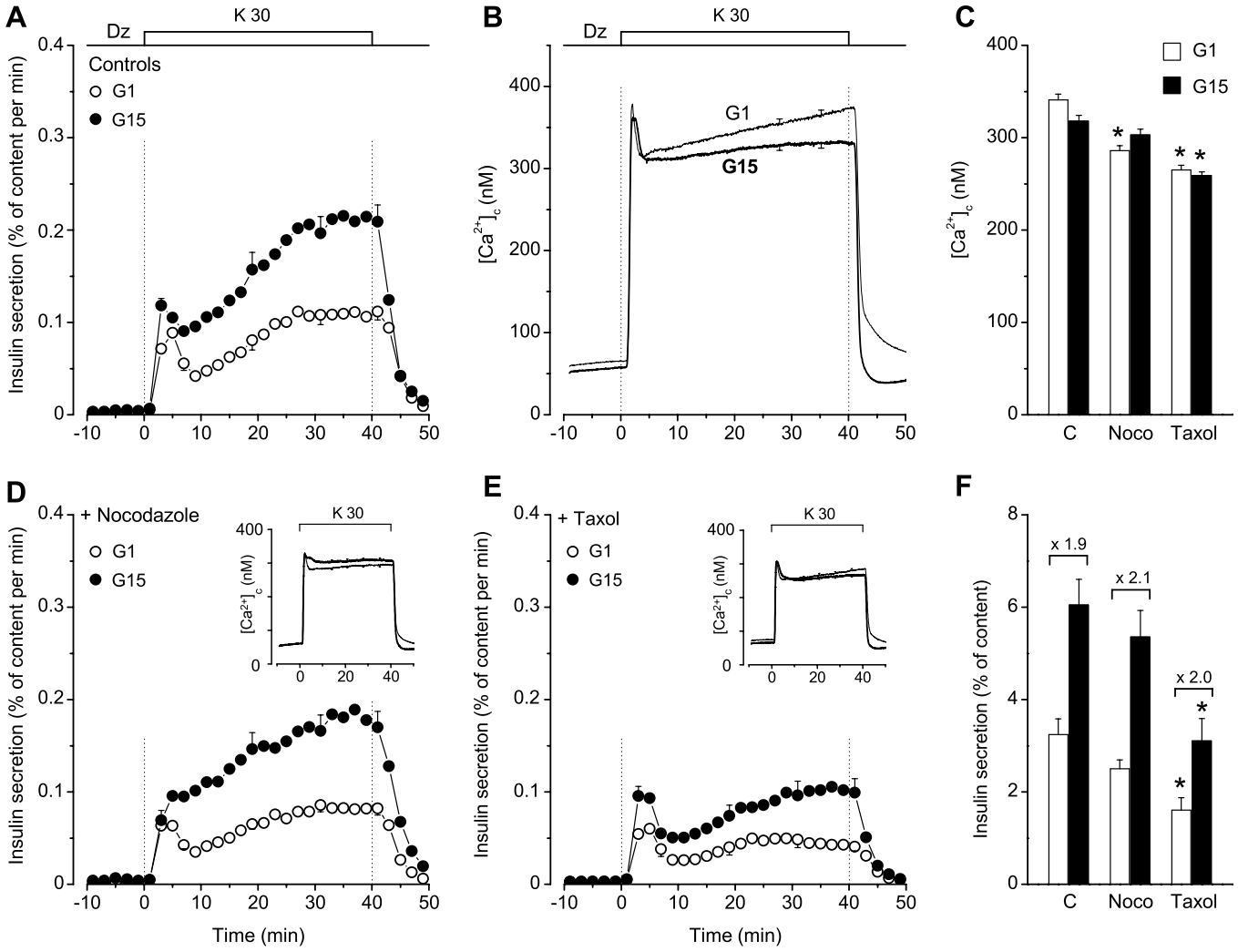

Fig. 2. Effects of tubulin depolymerization and polymerization on glucose amplification of insulin secretion during clamping of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in islets with KCl. Islets were first preincubated for 90 min with or without 10 μM Noco or 5 μM taxol. They were then used to measure insulin secretion (*A*, *D*, *E*, and *F*) or $[Ca^{2+}]_c$ (*B*, *C*, and *insets* in *D* and *E*). Experiments were performed in the presence of 100 μM diazoxide (Dz) to prevent any effect of glucose on ATP-sensitive potassium (K_{ATP}) channels and either 1 or 15 mM glucose (G1 or G15). Islets were stimulated by a rise of the KCl concentration from 4.8 to 30 mM (K30) at 0 min. Noco and taxol were present throughout the experiments. A, *B*, *D*, and *E* (and *insets*): time course of insulin and $[Ca^{2+}]_c$ changes induced by KCl. *C* and *F*: $[Ca^{2+}]_c$ and insulin responses integrated over the 40 min of stimulation. *Significant difference ($P < 0.05$ or less) with controls. Significant differences ($P < 0.001$) between responses in G1 and G15 are shown above pairs of columns, together with the fold-difference in insulin secretion (*F*). Values are means ± SE for 10 experiments of insulin secretion and 36–45 islets from 6 to 7 preparations for $[Ca^{2+}]_c$.

decrease due to uptake of Ca^{2+} in the endoplasmic reticulum (12, 19). In response to this rise in $[Ca^{2+}]_c$, biphasic insulin secretion occurred (Fig. 4*E*, filled circles), which was also delayed compared with the response to tolbutamide. Importantly, the effect of glucose on $[Ca^{2+}]_c$ was smaller than that of tolbutamide during both first and second phases (Fig. 4*C*, compare filled with open columns), whereas its effect on insulin secretion was more than twofold larger, also during both phases (Fig. 4*F*). This confirms that the amplifying action of glucose is a rapid and sustained phenomenon (39).

Islet treatment with nocodazole affected neither time course nor amplitude of tolbutamide- and glucose-induced $[Ca^{2+}]_c$ and insulin responses (Fig. 4), so that the amplifying action of glucose on insulin secretion remained unaltered (Fig. 4*F*). Islet treatment with taxol slightly attenuated the rise in $[Ca^{2+}]_c$ (Fig. 4, *A–C*) and the insulin secretion response (Fig. 4, *D–F*) produced by the two secretagogues. The effect on $[Ca^{2+}]_c$ was smaller (~10%) than that on insulin (~30%). However, despite this partial inhibition, the amplifying action of glucose on secretion was not altered (Fig. 4*F*). Altogether these results show that metabolic amplification does not depend on functional microtubules.

Impact of concomitant perturbation of microtubule and microfilament functions on metabolic amplification of insulin secretion. We recently reported that, while increasing insulin secretion, depolymerization of actin microfilaments did not impair metabolic amplification (39). We now investigated the impact of a concomitant inactivation of microtubules and microfilaments (Fig. 5). Islet microfilaments were disrupted by treatment with either cytochalasin B or latrunculin B (39). The lack of effect of these two drugs on tubulin polymerization has been described above (Fig. 1). We also ascertained that taxol and nocodazole had no effect on microfilaments. The proportion of depolymerized to polymerized actin (76%/24% in

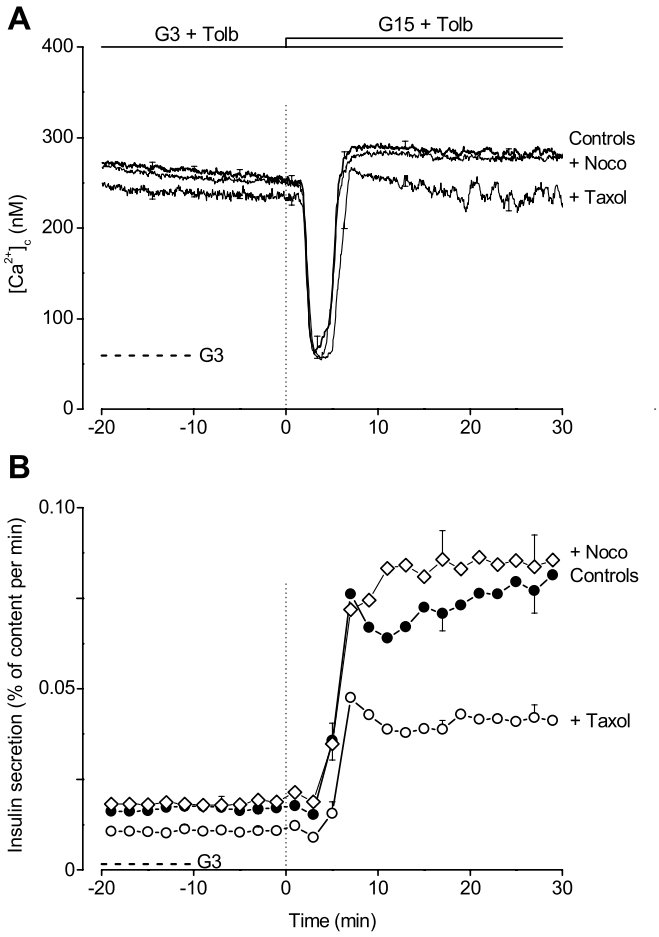

Fig. 3. Effects of tubulin depolymerization and polymerization on glucose amplification of insulin secretion during islet $[Ca^{2+}]_c$ elevation by tolbutamide (Tolb). Islets were first preincubated for 90 min with or without 10 μM Noco or 5 μM taxol. They were then used to measure $[Ca^{2+}]_c$ (*A* and *C*) or insulin secretion (*B* and *D*). Islets were stimulated with 500 μM Tolb in the presence of 3 mM glucose (G3) from −30 min, but only the last 20 min of this period are shown. The concentration of glucose was then raised to 15 mM (G15) at 0 min. Noco and taxol were present throughout the experiments. *A* and *B*: time course of $[Ca^{2+}]_c$ and insulin secretion changes in control islets and islets treated with Noco or taxol. The dotted line labeled G3 shows $[Ca^{2+}]_c$ and insulin secretion in the absence of Tolb. *C* and *D*: $[Ca^{2+}]_c$ and insulin responses integrated over the last 20 min in G3 + Tolb (−20–0 min) and in G15 + Tolb (10–30 min). *Significant difference ($P < 0.01$) with controls. Significant differences ($P < 0.01$ or less) between values in G3 and G15 are shown above pairs of columns, together with the fold-change in insulin secretion induced by high glucose (*D*). Values are means ± SE for 8–10 experiments of insulin secretion and 30–40 islets from 5 to 7 preparations for $[Ca^{2+}]_c$.

control islets) was unaffected by taxol (78%/22%) or nocodazole (75%/25%) alone. Neither drug impaired the depolymerizing action of cytochalasin B (96%/4%) or latrunculin B (99%/1%).

In a first series, we tested the impact of a concomitant interference with microtubules and microfilaments. Islets were preincubated and then perifused with 10 μM cytochalasin B and 5 μM taxol, whereas controls were preincubated and perifused without drug or with only one of the two drugs (Fig. 5, *A–D*). When tested alone, cytochalasin B increased tolbutamide- and glucose-induced insulin secretion approximately fourfold (Fig. 5, *A–C*), despite a slight inhibition of $[Ca^{2+}]_c$ in the presence of glucose (Fig. 5*D*). When compared with untreated controls, the effect of cytochalasin B on insulin secretion induced by both secretagogues was slightly attenuated by taxol (increase by 3-fold only) (Fig. 5, *A* and *B*), probably because of an inhibition of the $[Ca^{2+}]_c$ rise (Fig. 5*D*). However, when the combined effects of taxol + cytochalasin B were compared with the inhibited response of islets treated with taxol alone, the difference was fourfold again.

In a second series, we interfered with microtubules before depolymerizing microfilaments. Islets were preincubated without drug or with 10 μM nocodazole to disrupt microtubules and then perifused with nocodazole, 1 μM latrunculin B, or both (Fig. 5, *E–H*). In islets preincubated without drug, latrunculin B alone increased tolbutamide- and glucose-induced insulin secretion approximately fourfold (Fig. 5, *E–G*) without affecting $[Ca^{2+}]_c$ (Fig. 5*H*). Similar increases in insulin secretion by latrunculin B were observed in islets pretreated with nocodazole. When islets were preincubated with taxol alone and then perifused with taxol and cytochalasin B, results were similar to those shown in Fig. 5, *A–C*, when both drugs were present during the preincubation period (data not shown). We can thus conclude that microtubules are unnecessary to sustain the high rates of insulin secretion occurring when the brake exerted by actin microfilaments is lifted.

As to the specific question addressed in the study, these results clearly establish that amplification of insulin secretion, estimated by the ratio of the responses to glucose versus tolbutamide, is neither augmented nor impaired by concomitant inactivation of microfilaments and microtubules in β-cells (Fig. 5, *C* and *G*). The same conclusion can also be reached from experiments similar to those shown in Fig. 3. Raising the concentration of glucose from 3 to 15 mM in the presence of tolbutamide augmented insulin secretion more than fourfold in islets treated with cytochalasin B + taxol or with latrunculin B + nocodazole (not illustrated).

DISCUSSION

Our results show that β-cell microtubules play little role in short-term control of insulin secretion in mouse islets and, more specifically, are not implicated in metabolic amplification. They also provide further support to our

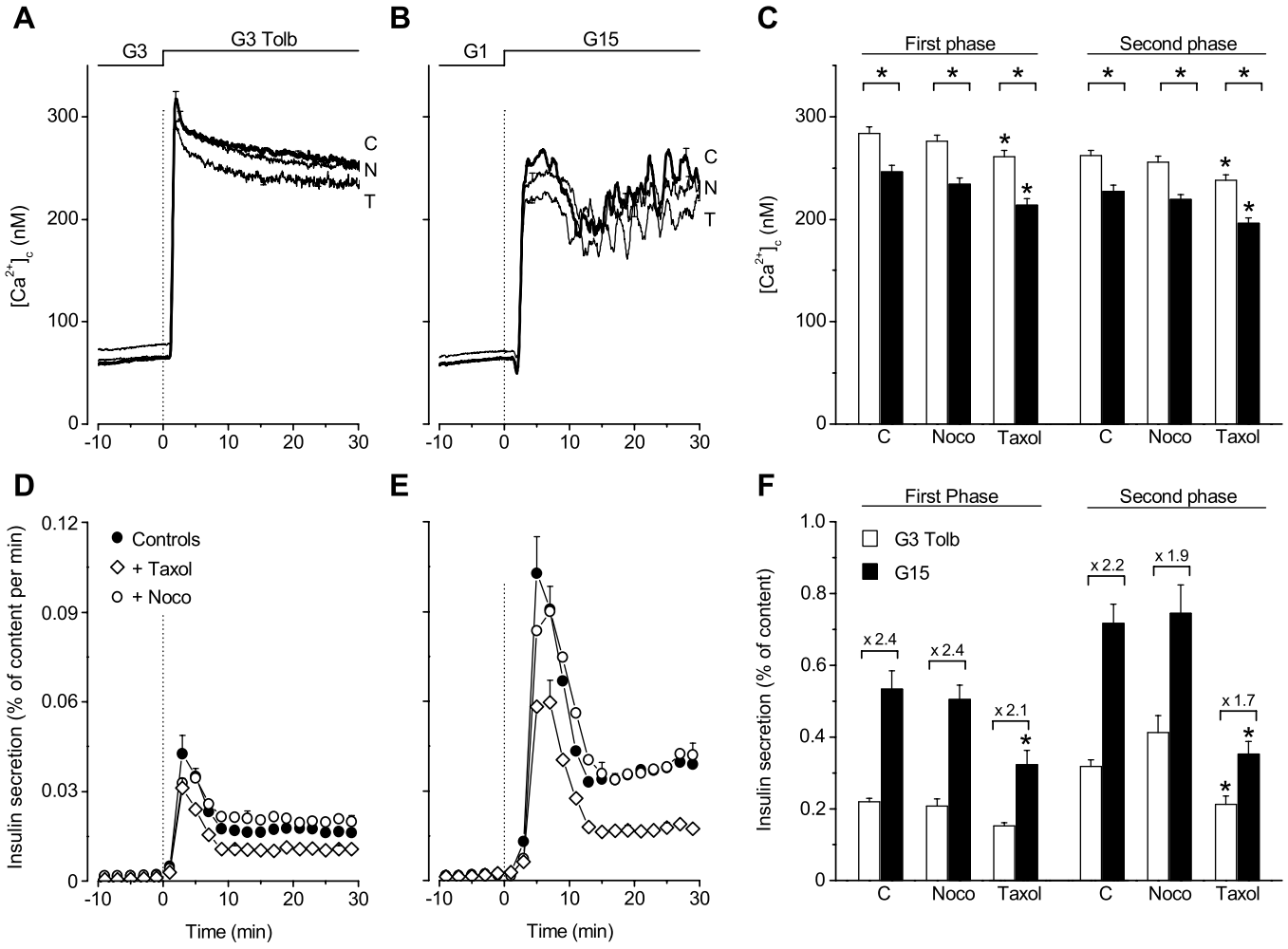

Fig. 4. Effects of tubulin depolymerization and polymerization on glucose- and Tolb-induced insulin secretion. Islets were first preincubated for 90 min with or without 10 μM Noco or 5 μM taxol. They were then used to measure $[Ca^{2+}]_c$ (*A–C*) or insulin secretion (*D–F*). Noco or taxol was present throughout the indicated experiments. *A* and *D*: islets were stimulated with 500 μM Tolb in the presence of G3. *B* and *E*: islets were stimulated by an increase in the glucose concentration from 1 to 15 mM. *A*, *B*, *D*, and *E*: time course of $[Ca^{2+}]_c$ and insulin changes. *C* and *F*: $[Ca^{2+}]_c$ and insulin responses integrated over 7 min for first phase (2–9 min for Tolb stimulation and 3–10 min for glucose stimulation) and 20 min for second phase (10–30 min for both). *Significant difference ($P < 0.01$) with controls. Significant differences ($P < 0.01$) between responses to Tolb in G3 (open bars) and to G15 (filled bars) are shown above pairs of columns, together with the fold-difference in insulin secretion (*C* and *F*).Values are means ± SE for 8–11 experiments of insulin secretion and 33–45 islets from 5 to 7 preparations for $[Ca^{2+}]_c$.

recent conclusions that amplification is a late event in stimulus-secretion coupling and influences both phases of secretion (39).

Experimental tools. Vinca alkaloids, such as vincristine and vinblastine, have previously been used to disrupt microtubules in β-cells and generally found to inhibit insulin secretion (8, 33, 34, 38). Except for the report that vincristine reduced the synchrony of $[Ca^{2+}]_c$ changes in clusters of MIN6 cells stimulated by tolbutamide or glucose (52), possible effects of this and related drugs on β-cell $[Ca^{2+}]_c$ have not been looked for. We found that vinblastine depolymerized tubulin and inhibited insulin secretion in mouse islets as expected, but also that it inhibited glucose- and KCl-induced $[Ca^{2+}]_c$ increases. We therefore elected to use another established tubulin-depolymerizing agent (28) nocodazole, which also completely disrupted islet microtubules but had virtually no effect on $[Ca^{2+}]_c$. Unlike vinblastine, nocodazole did not inhibit insulin secretion induced by three secretagogues, which suggests that previously observed inhibitions were at least partly secondary to changes in $[Ca^{2+}]_c$. We acknowledge, however, that nocodazole was reported to inhibit glucose-induced insulin secretion in rat islets (26) and HIT cells (14).

Although taxol is the most widely used agent to stabilize microtubules (29), its effects on β-cell function have only rarely been studied. Addition of taxol simultaneously with high glucose did not affect insulin secretion in HIT cells (14), but pretreatment with the drug inhibited glucose-induced insulin secretion in rat islets (26). Here, we found that pretreatment with taxol completely stabilized microtubules in mouse islets, inhibited insulin secretion induced by KCl, tolbutamide, or glucose, but also attenuated the rise in $[Ca^{2+}]_c$ produced by the three secretagogues. Microtubule-independent effects of taxol on Ca^{2+} release from the endoplasmic reticulum have been described in neural cells and cardiomyocytes (3, 62), but we are not aware of effects on voltage-dependent calcium channels. Whatever the underlying mechanism, the small decrease of $[Ca^{2+}]_c$ pro-

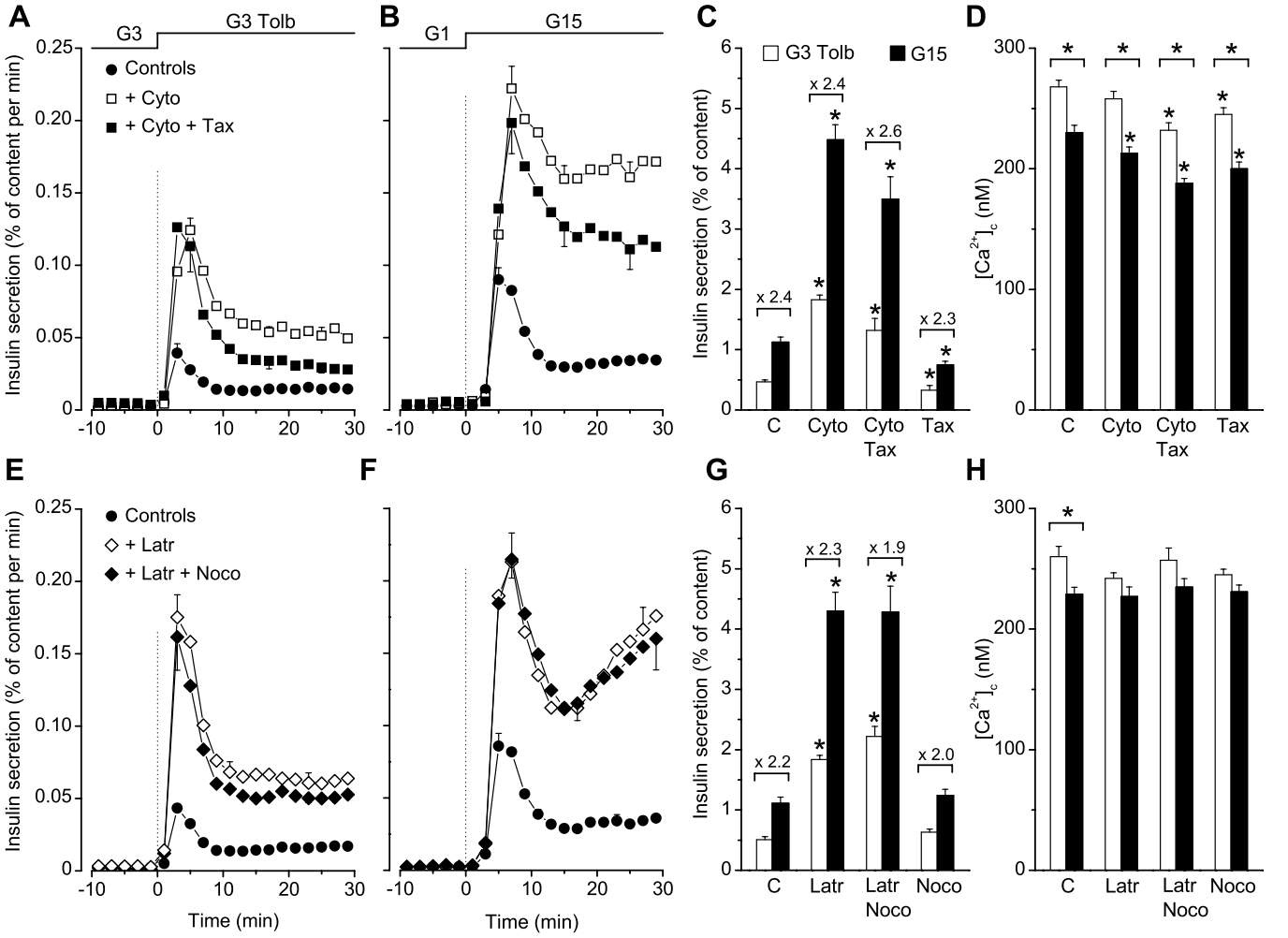

Fig. 5. Effects of combined inactivation of microtubules and microfilaments on glucose- and Tolb-induced insulin secretion. *A–D*: islets were first preincubated for 90 min with or without 10 μM Cyto and 5 μM taxol. They were then perifused in the presence of the drugs to measure insulin secretion or $[Ca^{2+}]_c$. *A* and *B*: time course of insulin secretion changes induced by Tolb in G3 or by an increase in the glucose concentration from 1 to 15 mM (G15). *C*: insulin responses integrated over 30 min. *D*: $[Ca^{2+}]_c$ responses integrated over 30 min. For clarity, the effect of taxol alone is shown only as integrated changes; its time course was similar to that shown in Fig. 4. *E* and *F*: similar experiments using the combination of 10 μM Noco and 1 μM Latr. The only difference was that Latr was not present during the preincubation period with or without Noco but added only 40 min before stimulation with Tolb or glucose. *Significant difference ($P < 0.01$) with controls. Significant differences ($P < 0.01$) between responses to Tolb in G3 (open bars) and to G15 (filled bars) are shown above pairs of columns, together with the fold-difference in insulin secretion (*C* and *G*). Values are means ± SE for 7–8 experiments of insulin secretion and 30–45 islets from 5 to 7 preparations (*D*) or 20–22 islets from 3 preparations (*H*) for $[Ca^{2+}]_c$.

duced by taxol probably contributed to the partial inhibition of secretion.

Microtubules and metabolic amplification. The major aim of the present study was to test the possibility that metabolic amplification of insulin secretion involves the microtubular system of β-cells. Our results refute the tested hypothesis. Amplification, defined as a larger secretion rate in face of a similar or lower islet $[Ca^{2+}]_c$, was not impaired by disruption or stabilization of microtubules. Importantly, neither the magnitude nor the rapidity of onset of amplification was altered by interference with microtubule function. Even when absolute levels of secretion were slightly reduced by taxol (an effect that we partly ascribe to inhibition of $[Ca^{2+}]_c$), the relative change caused by amplification was unaffected. Metabolic amplification has long been regarded as a process that specifically contributes to sustain second phase of insulin secretion (4, 47, 53, 59), but our recent data have shown that it is a rapid phenomenon that expresses itself within just a few minutes of exposure to high glucose (22, 24, 39). This can be appreciated under control conditions (Fig. 4) where first-phase insulin secretion is larger in response to glucose than to tolbutamide in face of a lower triggering $[Ca^{2+}]_c$ signal. This can also be seen when the concentration of glucose is increased in the presence of a saturating concentration of tolbutamide (Fig. 3). Preservation of both rapidity and magnitude of amplification after islet treatment with nocodazole and taxol implies that the process neither involves an acute action of glucose on microtubules nor is conditioned by a microtubule-mediated particular organization of pools of insulin granules.

Metabolic amplification, microtubules, and pools of insulin granules. The ~10,000 granules present in rodent β-cells (7, 43, 55) are functionally and/or geographically distributed into pools: a small pool of 50–100 readily releasable granules, a pool of ~700 granules docked (tethered) to the plasma membrane, a pool of ~1,500 almost docked granules (near but not attached to the plasma membrane), and a large reserve pool of

~8,000 granules. Recent studies, using the total internal reflection fluorescence (TIRF) technique, have discovered that, contrary to classic theories (47), granules must not necessarily dock before entering the releasable pool and undergoing exocytosis ("newcomer granules") (31, 42, 49).

Models implicating microtubules in metabolic amplification view the amplification process as an accelerated refilling of the pool of readily releasable granules through mobilization of insulin granules along microtubules (13, 58). Early cinematographic studies of primary β-cells in monolayer cultures showed that glucose increased saltatory movements of insulin granules and that these movements were inhibited by vinblastine, vincristine, or colchicine (30, 32, 50). More recent studies imaged cell lines in which insulin granules were tagged with a targeted fluorescent protein (20, 27, 59). This improved technique more clearly distinguished between slow random movements of the majority of granules and fast directed movements of a small population of granules. Stimulation of Ca^{2+} influx by either glucose or KCl selectively augmented directed granular movements, an effect that was suppressed by disruption of microtubules with nocodazole (20) or by inhibition of the microtubule-associated kinesin (58, 59). These observations prompted the proposal that these fast, microtubule-dependent movements serve to replenish the readily releasable pool of granules (20) and that their acceleration by glucose corresponds to the amplification process (58).

From our measurements of insulin secretion rates and insulin content of the islets, we can calculate, on the basis of 10,000 granules per cell, the number of granules released by each β-cell (39). We estimate that, over 30 min of stimulation, each β-cell released about 50 granules in response to tolbutamide in low glucose and released about 115 granules in response to high glucose. These numbers are similar to those of granules in the readily releasable pool but much smaller than those in the pools (be they docked or almost docked) that serve to refill the releasable pool. After disruption of the web of actin microfilaments with either cytochalasin B or latrunculin B, the numbers of released granules markedly increased to about 185 (tolbutamide) and 420 (high glucose). Importantly, these three- to fourfold increases also occurred in the absence of functional microtubules. Altogether, our results obtained in whole islets indicate that intact microtubules are not required for acute refilling of the releasable pool and for short-term control of insulin secretion. Interestingly, estimations of exocytosis by capacitance measurements in mouse β-cells treated with colcemid (45) or INS1 cells treated with vindesin (27) also suggested that microtubules are not necessary for replenishment of the readily releasable pool of insulin granules after its emptying by trains of depolarizations. Moreover, we can exclude that the microtubule-dependent acceleration of fast granular movements by glucose (20, 58) underlies metabolic amplification for a simple reason. Unlike insulin secretion (Fig. 2) (21), this acceleration of granular movements was not augmented further by high glucose in the presence of KCl (20). Kinesin-driven translocation of insulin granules along microtubules (9, 37, 58) probably contributes to maintenance of long-term β-cell secretory function but is unlikely to play an active role in acute regulation of insulin secretion, except perhaps in pathological degranulated β-cells or in inherently poorly granulated cell lines. Other functions of kinesin-1 are also possible. Thus targeted inactivation of kinesin-1 in β-cells led to inhibition of both phases of glucose-induced insulin secretion in vivo, independently of any alteration of the subcellular localization of insulin granules (6).

In conclusion, in sharp contrast with the increase in insulin secretion observed after disruption or stabilization of actin microfilaments (39, 57, 60), no acute secretory changes follow alterations of microtubule function in primary mouse β-cells. Together, our previous (39) and the present study show that metabolic amplification does not involve recruitment of reserve granules by the cytoskeleton. In agreement with a previously proposed model (54), we reinforce our recent conclusion that metabolic amplification corresponds to acceleration of the priming process conferring release competence to insulin granules (39). This should now help identifying the molecular mechanisms and cellular effectors of a quantitatively important (22) regulatory step of stimulus-secretion coupling in β-cells.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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Role of the beta-cell cytoskeleton in metabolic and neurohormonal amplification of insulin secretion

Nizar MOURAD

Background: Glucose-induced insulin secretion is controlled by two pathways that depend on beta-cell metabolism. The *triggering pathway* starts by a rise in the cytosolic ATP/ADP ratio and closure of ATP-sensitive K^+ channels in the plasma membrane, which leads to depolarization, opening of voltage-gated Ca^{2+} channels, influx of Ca^{2+} and an increase of cytosolic $[Ca^{2+}]_c$ which induces exocytosis of insulin granules. In parallel a *metabolic amplifying pathway* augments the secretory response to Ca^{2+} via messengers and effectors that remain undetermined. Insulin secretion is also regulated by neural and hormonal signals which also mainly act as amplifying factors, i.e., they increase the secretory response triggered by glucose or another stimulus without affecting $[Ca^{2+}]_c$. I first investigated the involvement of beta-cell microfilaments and microtubules in metabolic amplification during the two phases of insulin secretion. I then evaluated whether beta-cell microfilaments are required for neurohormonal amplification and, simultaneously reevaluated the involvement of cAMP and PKC in metabolic amplification.

Methods: Isolated mouse islets were cultured overnight before being treated with cytoskeleton-disrupting drugs and then perifused to measure insulin secretion and monitor $[Ca^{2+}]_c$ changes. The proportions of free and polymerized actin and tubulin were determined in islet protein extracts. Metabolic amplification was mainly studied by comparing the Ca^{2+} and secretory responses to glucose and tolbutamide, a drug that mimics the triggering but not the amplifying effect of glucose.

Results: I first established that metabolic amplification is involved in both phases of insulin secretion and not only in the second phase as suggested in most current models. I also found that both microfilament depolymerization and stabilization increased the secretory response, and that metabolic amplification persisted in the absence of functional microfilaments. My results then showed that disruption of microtubules does not affect insulin secretion whereas their stabilization slightly inhibits it partly because of a decrease in $[Ca^{2+}]_c$. Most importantly, functional microtubules proved unnecessary for metabolic amplification even during simultaneous disruption of microfilaments. In a third study, I established that amplification of insulin secretion by cAMP does not require actin microfilaments and that metabolic amplification is not mediated by cAMP. My fourth study showed that microfilaments are dispensable for amplification of insulin secretion by protein kinase C activation and I confirmed that the kinase is not involved in metabolic amplification.

Conclusions: Metabolic amplification of insulin secretion is a rapid phenomenon involved in both phases of glucose-induced insulin secretion. It does not involve facilitation of insulin granule mobilization from reserve pools by the beta-cell cytoskeleton but affects a very distal step which I speculate to be acceleration of the priming of insulin granules possibly belonging to a highly Ca^{2+}-sensitive pool. Neurohormonal amplification of insulin secretion is distinct from metabolic amplification (cAMP and PKC are not the mediators of the amplifying effect of glucose), but probably affects the same steps of insulin granule exocytosis independently of beta-cell microfilaments.