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Oscillatory Ca^{2+} Signaling in Glucose-stimulated Murine Pancreatic β-Cells

Modulation by Amino acids, Glucagon, Caffeine and Ryanodine

BY

MEFTUN AHMED

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ABSTRACT

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Oscillations in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) is the key signal in glucose-stimulated β-cells governing pulsatile insulin release. The glucose response of mouse β-cells is often manifested as slow oscillations and rapid transients of $[Ca^{2+}]_i$. In the present study, microfluorometric technique was used to evaluate the role of amino acids, glucagon, ryanodine and caffeine on the generation and maintenance of $[Ca^{2+}]_i$ oscillations and transients in individual murine β-cells and isolated mouse pancreatic islets. The amino acids glycine, alanine and arginine, at around their physiological concentrations, transformed the glucose-induced slow oscillations of $[Ca^{2+}]_i$ in isolated mouse β-cells into sustained elevation. Increased Ca^{2+} entry promoted the reappearance of the slow $[Ca^{2+}]_i$ oscillations. The $[Ca^{2+}]_i$ oscillations were more resistant to amino acid transformation in intact islets, supporting the idea that cellular interactions are important for maintaining the oscillatory activity. Individual rat β-cells responded to glucose stimulation with slow $[Ca^{2+}]_i$ oscillations due to periodic entry of Ca^{2+} as well as with transients evoked by mobilization of intracellular stores. The $[Ca^{2+}]_i$ oscillations in rat β-cells had a slightly lower frequency than those in mouse β-cells and were more easily transformed into sustained elevation in the presence of glucagon or caffeine. The transients of $[Ca^{2+}]_i$ were more common in rat than in mouse β-cells and often appeared in synchrony also in cells lacking physical contact. Depolarization enhanced the generation of $[Ca^{2+}]_i$ transients. In accordance with the idea that β-cells have functionally active ryanodine receptors, it was found that ryanodine sometimes restored oscillatory activity abolished by caffeine. However, the IP_3 receptors are the major Ca^{2+} release channels both in β-cells from rats and mice. Single β-cells from *ob*/*ob* mice did not differ from those of lean controls with regard to frequency, amplitudes and half-widths of the slow $[Ca^{2+}]_i$ oscillations. Nevertheless, there was an excessive firing of $[Ca^{2+}]_i$ transients in the β-cells from the *ob*/*ob* mice, which was suppressed by leptin at close to physiological concentrations. The enhanced firing of $[Ca^{2+}]_i$ transients in *ob*/*ob* mouse β-cells may be due to the absence of leptin and mediated by activation of the phospholipase C signaling pathway.

Key Words: pancreatic β-cells, islets of Langerhans, glucose, Ca^{2+} oscillations, Ca^{2+} transients, potassium, ryanodine, caffeine, glucagon, cAMP, inositol 1,4,5-trisphosphate, fura-2, glycine, alanine, arginine, Ca^{2+} channels, rats, lean mice, *ob*/*ob* mice, leptin.

Meftun Ahmed, Department of Medical Cell Biology, Uppsala University, Biomedicum, Box 571, SE-751 23 Uppsala, Sweden

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To

me', sumon

Did you know freedom exists
in a school book
Did you know madmen are
running our prison
… … … … …… … … …
We're perched headlong
On the edge of boredom
We're reaching for death
On the end of a candle
We're trying for something
That's already found us

Jim Morrison; An American Prayer

REPORTS CONSTITUTING THE THESIS

This thesis is based on the following papers, which in the text will be referred to by their Roman numerals:

I. **Ahmed M, Grapengiesser E, Hellman B**. Amino acid transformation of oscillatory Ca^{2+} signals in mouse pancreatic β-cells. *J Endocrinol* 160: 191-5, 1999.

II. **Ahmed M, Grapengiesser E**. Pancreatic β-cells from obese-hyperglycemic mice are characterized by excessive firing of cytoplasmic Ca^{2+} transients. *Endocrine* 15: 57-62, 2001.

III. **Ahmed M, Grapengiesser E**. Ca^{2+} handling of rat pancreatic β-cells exposed to ryanodine, caffeine and glucagon (manuscript).

CONTENTS

ABBREVIATIONS

ADP	adenosine 5´-diphosphate
ATP	adenosine 5´-triphosphate
cADPR	cyclic adenosine 5´-diphosphate ribose
cAMP	cyclic adenosine 3´5´-monophosphate
$[Ca^{2+}]_i$	cytoplasmic Ca^{2+} concentration
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ER	endoplasmic reticulum
FKBP	FK506 binding protein
GSIS	glucose-stimulated insulin secretion
Hepes	N-(2-hydroxyethyl)piperazine-N´-(2-ethanesulfonic acid)
IP_3	inositol 1,4,5-trisphosphate
IP_3R	inositol 1,4,5-trisphosphate receptor
JAK-STAT	janus kinase signal transducer and activator of transcription
K_{ATP} channel	ATP-sensitive potassium channel
MOPS	3-(N-morpholino)propanesulfonic acid
NAD^+	nicotinamide adenine dinucleotide
NAD(P)H	nicotinamide adenine dinucleotide phosphate
Ob-R	leptin receptor
Ob-Rb	long isoform of leptin receptor
PI	phosphoinositide
PLC	phospholipase C
RyR	ryanodine receptor
SERCA	sarco-endoplasmic reticulum Ca^{2+} ATPase
TRP	transient receptor potential
VDCC	voltage dependent Ca^{2+} channel

INTRODUCTION

The pancreatic β-cells are 'fuel sensors' that integrate the signals from different nutrients, incretins, peptide hormones and neurotransmitters and release insulin in a pulsatile manner. This pattern of insulin secretion, under physiological conditions, keeps the blood glucose concentration at around 5 mM in fasting mammals, including humans. Among the factors affecting insulin secretion, glucose is the most important physiological stimulus and considered as the primary regulatory signal because of its 'initiator' property and being required for the potentiating effect of other signals (Hedeskov, 1980). Until recently the exact sequence of biochemical events involved in glucose-stimulated insulin secretion (GSIS) from the pancreatic β-cells has not been identified. Nevertheless, it is well established that an increase in the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) is a crucial step (Hellman *et al.*, 1971; Wollheim & Sharp, 1981; Hellman *et al.*, 1994).

The most widely accepted model of GSIS schematizes that glucose after entering β-cells through a high capacity glucose transporter, is metabolized and provides a complex cascade of stimulatory signals for insulin secretion. The glucose-phosphorylating enzyme, glucokinase, is central for glucose metabolism in pancreatic β-cells. It is conceptualized as the 'glucose sensor' in β-cells, and is rate limiting, since increased glucose phosphorylation by glucokinase enhances glycolysis, glucose oxidation and eventually increases the rate of ATP generation (Matschinsky *et al.*, 1998). The increased ATP production raises the ATP/ADP ratio and causes closure of ATP-sensitive K^+ (K_{ATP}) channels resulting in membrane depolarization, opening of the voltage dependent Ca^{2+} channels (VDCCs) and subsequent entry of Ca^{2+} from extracellular space (Ashcroft & Rorsman, 1989). Glucose has a dual action on $[Ca^{2+}]_i$ in both stimulating the entry and sequestration of the ion (Gylfe, 1988). When the entry of Ca^{2+} is suppressed by lowering of its extracellular concentration or by blocking the VDCC, the net effect of glucose is a lowering of $[Ca^{2+}]_i$ (Gylfe, 1988; Hellman *et al.*, 1994). However, glucose can also temporarily increase $[Ca^{2+}]_i$ by promoting mobilization of Ca^{2+} from the endoplasmic reticulum (ER; Roe *et al.*, 1993; Liu *et al.*, 1996; Jijakli & Malaisse, 1998). The rise of $[Ca^{2+}]_i$ acts as the primary intracellular messenger

that couples physiological or pharmacological secretagogues to exocytosis of the insulin-containing granules. A characteristic feature of the $[Ca^{2+}]_i$ response to glucose is its oscillatory nature observed both in individual β-cells (Grapengiesser *et al.*, 1988; Wang & McDaniel, 1990) and in intact pancreatic islets (Valdeolmillos *et al.*, 1989; Liu *et al.*, 1998). Studies on individual β-cells have revealed that glucose induces slow oscillations of $[Ca^{2+}]_i$ with frequencies of 0.05-0.5/min (Hellman *et al.*, 1992) and these oscillations explain the pulsatile nature of insulin release (Bergsten *et al.*, 1994; Gylfe *et al.*, 2000), the impairment of which is an early phenomenon in the development of both type 1 (Bingley *et al.*, 1992) and type 2 (O'Rahilly *et al.*, 1988) diabetes.

Entry of Ca^{2+} into the β-cells

Patch clamp studies have characterized two types of voltage-dependent Ca^{2+} channels in pancreatic β-cells with properties similar to those of L- and T-type channels in other cells (Ashcroft & Rorsman, 1989). In the mouse β-cells the voltage-activated Ca^{2+} current has been reported to be carried only by the L-type Ca^{2+} channels (Rorsman *et al.*, 1988), whereas rat (Satin & Cook, 1988; Ashcroft *et al.*, 1990) and human β-cells (Misler *et al.*, 1992) and the rat-derived RINm5F cells (Findlay & Dunne, 1985) exhibit both L- and T-type Ca^{2+} currents. The L-type channel is distinguished from T-, N-, P- and R-type Ca^{2+} channels by its sensitivity to the high-affinity, voltage-dependent blocking properties of dihydropyridines, phenylalkylamines, such as methoxyverapamil; and benzothiazepines (Hockerman *et al.*, 1997). Opening of L-type Ca^{2+} channels is greatly enhanced by dihydropyridine Ca^{2+} channel agonists like Bay K8644 whereas other types of Ca^{2+} channels are not significantly affected (Tsien *et al.*, 1988). T-type Ca^{2+} channels are activated by depolarization to potentials positive to -50 mV and inactivates rapidly at -40 mV. The channel remains partially inactivated at the resting potential of -70 mV (Ashcroft & Rorsman, 1989; Sala & Matteson, 1990). Recent studies have shown that rat β-cells also exhibit ω-conotoxin sensitive N-type (Ramanadham & Turk, 1994) and ω-agatoxin sensitive P-type (Ligon *et al.*, 1998) Ca^{2+} currents and proposed that they have a physiological role in excitation-secretion coupling. However, the predominant Ca^{2+}-current in pancreatic β-cells is carried by L-type channels

(Rorsman *et al.*, 1994). Both glucose-induced rise of $[Ca^{2+}]_i$ (Arkhammar *et al.*, 1989; Grapengiesser *et al.*, 1989a; Ramanadham & Turk, 1994) and insulin release (Wollheim & Pozzan, 1984; Al Mahmood *et al.*, 1986; Ohta *et al.*, 1993) are consequently blocked by inhibitors of this Ca^{2+} channel.

Apart from the regulation by voltage, the L-type Ca^{2+} channels are modulated by glucose metabolism (Smith *et al.*, 1989), guanine nucleotides (Béguin *et al.*, 2001), protein phosphorylation (Jones & Persaud, 1998) and by Ca^{2+} itself (Rorsman & Trube, 1986). The α_1 subunit of VDCC contains plausible sites for phosphorylation by protein kinases A and C (Wheeler *et al.*, 1994). In β-cells phosphorylation by cAMP-dependent protein kinase A increases Ca^{2+} influx by potentiating the activation and inhibiting the inactivation of L-type Ca^{2+} channels (Ämmälä *et al.*, 1993; Kanno *et al.*, 1998). A store-operated pathway may also contribute to the influx of Ca^{2+} into β-cells (Liu & Gylfe, 1997; Miura *et al.*, 1997), perhaps through the members of the TRP (transient receptor potential) family of channel proteins (Sakura & Ashcroft, 1997).

Intracellular Ca^{2+} transport

In many cell types, including pancreatic β-cells, the average Ca^{2+} concentration is in the millimolar range whereas the resting $[Ca^{2+}]_i$ is maintained around 100 nM. Most of the cellular Ca^{2+} therefore remains either bound to membrane surfaces and cytosolic components or is accumulated within membrane-bound organelles (Meldolesi *et al.*, 1988). The major Ca^{2+} sequestrating organelles include endoplasmic reticulum, mitochondria and secretory granules (Hellman & Gylfe, 1986). There are scattered reports about a Ca^{2+} accumulating capacity of the Golgi complex (Pinton *et al.*, 1998), lysosomes (Haller *et al.*, 1996), endosomes (Gerasimenko *et al.*, 1998) and nucleus (Brini *et al.*, 1993). In pancreatic β-cells, the free Ca^{2+} in the ER is in the range of 200-500 μM (Tengholm *et al.*, 1999; Maechler *et al.*, 1999) and is maintained as a result of the steady-state balance of Ca^{2+} uptake and release. The uptake of Ca^{2+} is mediated by pumps, which belong to sarco-endoplasmic reticulum Ca^{2+} ATPases (SERCA). Ca^{2+} release can be triggered by the inositol 1,4,5-trisphosphate (IP_3) receptors and ryanodine receptors (RyR).

Role of SERCA pumps

The SERCA pump is an intrinsic membrane protein encoded by three genes SERCA-1, -2, and -3 (Carafoli & Brini, 2000). In pancreatic β-cells the SERCA-2b and SERCA-3 isoforms are co-expressed (Váradi *et al.*, 1996) and play major roles in Ca^{2+} homeostasis (Roe *et al.*, 1994a; Worley, III *et al.*, 1994). These pumps maintain a high free Ca^{2+} concentration within the ER lumen in equilibrium with Ca^{2+} bound to the ubiquitous luminal proteins, calreticulin and binding protein BiP (Meldolesi & Pozzan, 1998). Inhibition of the SERCA pump may result in multiple changes in cell function, including alterations in signaling, gene expression, Ca^{2+} entry, cell proliferation and apoptosis which can be attributed to a rise of $[Ca^{2+}]_i$ and/or depletion of intracellular Ca^{2+} pools (Hussain & Inesi, 1999). Refilling of the ER store by SERCA pumps is a key factor that controls the frequency of $[Ca^{2+}]_i$ oscillations depending on intracellular release. Recent studies have demonstrated that the loss of SERCA activities and the defects of SERCA-3 gene expression in β-cells are associated with human diabetes (Váradi *et al.*, 1999) and altered patterns of glucose-induced $[Ca^{2+}]_i$ changes in *db*/*db* mice (Roe *et al.*, 1994b), Goto-Kakizaki (Váradi *et al.*, 1996) and neonatal streptozotocin rats (Levy *et al.*, 1998; Marie *et al.*, 2001).

In certain cell types, evidence has been provided that SERCA pumps are also present in organelles other than the ER (Lanini *et al.*, 1992; Kovàcs *et al.*, 1997; Lee *et al.*, 1997; Misquitta *et al.*, 1999; Rojas *et al.*, 2000). However, such expression has so far not been reported in pancreatic β-cells, in which the SERCA isoforms are restricted to the ER membrane (Prentki *et al.*, 1984a; Wolf *et al.*, 1988). Acting specifically on the SERCA pumps, thapsigargin, a plant extract from the root of *Thapsia garganica*, induces specific emptying of the ER stores (Thastrup *et al.*, 1990; Kijima *et al.*, 1991). Recent studies have demonstrated that interaction of SERCA 2b with the ER proteins, calreticulin and calnexin regulates Ca^{2+} homeostasis including oscillations of $[Ca^{2+}]_i$ in nonmuscle cells (John *et al.*, 1998; Roderick *et al.*, 2000).

Role of IP_3 receptors

Release of Ca^{2+} from intracellular stores in response to activation of phospholipase C (PLC) is mediated by the interaction of IP_3 with its receptors (IP_3R) (Berridge & Irvine, 1984). At least three closely related subtypes (1, 2, 3) of the IP_3Rs, which assemble into both homo- and heterotetrameric complexes (Nucifora, Jr. *et al.*, 1996), are expressed in pancreatic β-cells from rats (Lee *et al.*, 1998), mice (Lee & Laychock, 2001) and insulinoma cell lines (De Smedt *et al.*, 1994). The ER is the organelle in which IP_3Rs are most abundantly expressed (Blondel *et al.*, 1993), however, in some cell types, IP_3Rs have been localized to the nucleus (Humbert *et al.*, 1996), secretory granules (Blondel *et al.*, 1994a) and plasma membrane (Tanimura *et al.*, 2000). IP_3Rs have been ambiguously demonstrated on the secretory granules membrane in pancreatic β-cells (Blondel *et al.*, 1994b; Ravazzola *et al.*, 1996; Nucifora, Jr. *et al.*, 1996; Srivastava *et al.*, 1999), but it seems unlikely that Ca^{2+} release from this organelle is involved in agonist-stimulated insulin secretion (Prentki *et al.*, 1984a; Prentki *et al.*, 1984b; Pouli *et al.*, 1998; Scheenen *et al.*, 1998). Mobilization of Ca^{2+} through IP_3Rs is regulated in a complex way by IP_3 (Marchant & Taylor, 1998), $[Ca^{2+}]_i$, the Ca^{2+} concentration in the ER (Taylor, 1998), nucleotides (Maes *et al.*, 2000), phosphorylation by protein kinases (Nakade *et al.*, 1994; LeBeau *et al.*, 1999) and various other modulators. In spite of the structural similarity between different IP_3R isoforms, they exhibit distinct functional properties (Hagar & Ehrlich, 2000), and the expression of each isoform is species and tissue specific (Newton *et al.*, 1994; De Smedt *et al.*, 1994) either with a single or a combination of IP_3R subtypes. Whereas the type 1 receptor is most abundant in mouse islets (Lee & Laychock, 2001), type 3 dominates in rat islets (Blondel *et al.*, 1993; Lee *et al.*, 1998). The relative expression of various isoforms in β-cells is transcriptionally regulated by glucose (Lee *et al.*, 1999), carbachol (Lee & Laychock, 2001) and cAMP (Lee & Laychock, 2000). Recent studies have demonstrated that the type 1 and type 3 receptors bind IP_3 with different affinities and are modulated differentially by Ca^{2+}, ATP and other regulators, suggesting that each IP_3R subtype plays a distinct role in β-cell signal transduction and insulin secretion. For example, type 3 IP_3R channel activity does not exhibit the bell-shaped dependence on

$[Ca^{2+}]_i$ as the type 1 isoform; instead its open probabilty increases monotonically with increased Ca^{2+} (Hagar *et al.*, 1998). Thus, Ca^{2+} release via type 3 IP_3R results in a positive feedback cycle, that leads to 'all or none' Ca^{2+} signaling. However, when composed of heterotetramers, the IP_3Rs tend to adopt the responses of the most sensitive and/or modulatable subunits (Miyakawa *et al.*, 1999; Swatton *et al.*, 1999).

Role of ryanodine receptors

The ryanodine receptors are encoded by three separate genes, *ryr-1*, *ryr-2*, and *ryr-3* (Sutko & Airey, 1996). Of the three isoforms only type 2 RyRs (RyR2s) are expressed on the ER membrane of pancreatic β-cells (Islam *et al.*, 1998; Holz *et al.*, 1999). Unlike IP_3 receptors, RyR2s are homotetramers of four RyR2 polypeptides (Franzini-Armstrong & Protasi, 1997), each of which binds one FK506 binding protein (FKBP12.6; Noguchi *et al.*, 1997) that stabilizes the channel (MacKrill, 1999) and facilitates coupled gating (Marx *et al.*, 1998). In addition to endogenous regulators including Ca^{2+}, Mg^{2+}, ATP and cyclic ADP ribose (cADPR), several pharmacological modulators, especially ryanodine, caffeine and the immunosuppressant drug FK506 influence the RyR-channel function (Shoshan-Barmatz & Ashley, 1998). Phosphorylation of RyRs is the underlying physiological mean, that increases the agonist responsiveness of the channel (Hain *et al.*, 1995). Ligands, known to open RyR channels (μM Ca^{2+}, caffeine), stimulate the binding of low concentrations of ryanodine (< 10 μM) to the high affinity site, which locks the channel in a partially open subconductance state (Buck *et al.*, 1992). As the concentration of ryanodine is increased, the affinity of RyRs for ryanodine decreases. Ryanodine (≥ 70 μM) produces a unidirectional transition from ½ to a ¼ conductance fluctuation, whereas ≥ 200 μM ryanodine causes complete closure of the channel enlightening the fact that ryanodine has an allosteric negative interaction among the four binding sites on RyRs (Buck *et al.*, 1992).

Cyclic ADPR, the natural modulator of RyRs, is synthesized from NAD^+ by the enzyme ADP-ribosyl cyclase and its mammalian homologues, CD38 and CD157 (Lee, 1999), which are expressed in various cell types including β-cells (Koguma *et al.*, 1994; Kajimoto *et al.*, 1996). Autoantibodies against CD38

have been detected in patients with both type 1 and type 2 diabetes (Ikehata *et al.*, 1998; Pupilli *et al.*, 1999; Antonelli *et al.*, 2001). In Japanese type 2 diabetic patients, mutation in the CD38 gene with a reduction of the enzyme activity has been described (Yagui *et al.*, 1998). When CD38 cyclase activity in mouse β-cells is inactivated by ADP-rybosylation, glucose-induced increase of cADPR, $[Ca^{2+}]_i$ and insulin secretion are impaired (An *et al.*, 2001). More intriguingly, CD38 knockout mice exhibit a similar aberration in glucose effects on β-cells (Kato *et al.*, 1999). The expression of CD38 is also decreased in animal models of diabetes such as GK rats (Matsuoka *et al.*, 1995) and *ob*/*ob* mice (Takasawa *et al.*, 1998). Despite all these experiments, the original proposal of cADPR as a Ca^{2+}-mobilizing second messenger in β-cells (Takasawa *et al.*, 1993) could not be confirmed by others (Islam *et al.*, 1993; Rutter *et al.*, 1994; Willmott *et al.*, 1995; Webb *et al.*, 1996; Malaisse *et al.*, 1997; Tengholm *et al.*, 1998), and different opinions have been expressed whether the RyRs play any significant role for Ca^{2+} release from intracellular stores. Recently, it has been suggested that activation of RyRs require cAMP-dependent phosphorylation (Islam *et al.*, 1998; Lemmens *et al.*, 2001) and they mediate a distinct 'context-dependent' Ca^{2+} signaling for insulin release (Islam & Lemmens, 2001).

Ca^{2+} signals in terms of slow oscillations and rapid transients

Glucose stimulation of individual β-cells produces changes in $[Ca^{2+}]_i$ kinetics, which are manifested as slow oscillations and rapid transients (Grapengiesser *et al.*, 1991). The slow oscillations usually appear at glucose concentrations of 7-20 mM with different thresholds for the individual cells (Hellman *et al.*, 1992). The oscillations have typical frequencies of 0.05-0.5/min, starting from a basal level of 60-90 nM with amplitudes of 300-500 nM. The initial β-cell response to glucose is a brief lowering of $[Ca^{2+}]_i$, due to sequestration of Ca^{2+} in intracellular compartments (Gylfe, 1988; Roe *et al.*, 1994a; Aizawa *et al.*, 1995), followed by a rise due to influx of Ca^{2+} (Grapengiesser *et al.*, 1989a). The slow $[Ca^{2+}]_i$ oscillations are elicited not only by glucose but also by leucine (Grapengiesser *et al.*, 1989b), isoleucine (Martin & Soria, 1995), α-keto-isocaproate (Martin *et al.*, 1995) and tolbutamide (Grapengiesser *et al.*, 1990)

and depend on extracellular Ca^{2+}. Blocking of VDCCs or lowering of extracellular Ca^{2+} to 0.8 mM results in disappearance of the oscillations (Hellman *et al.*, 1992). Parallel measurements of electrical activity and $[Ca^{2+}]_i$ show that the oscillations of $[Ca^{2+}]_i$ in isolated β-cells occur in synchrony with bursts of action currents and reflect variations in Ca^{2+} influx (Dryselius *et al.*, 1999). Using the activity of K_{ATP} channels as an indicator of the ATP concentration, it was found that this metabolite fluctuates with a frequency similar to that of the slow oscillations even in the presence of sub-stimulatory glucose concentrations (Dryselius *et al.*, 1994). Further evidence that cyclic variations of β-cell metabolism underlie rhythmical depolarization, resulting $[Ca^{2+}]_i$ oscillations is that there is a close correlation between changes in the ATP/ADP ratio and mitochondrial respiration and that the glucose-induced rise in the ATP/ADP ratio and mitochondrial NAD(P)H fluorescence precede the increase of $[Ca^{2+}]_i$ (Deeney *et al.*, 2000).

Under certain condition, glucose induces transients of $[Ca^{2+}]_i$ due to brief periods of Ca^{2+} influx (Eberhardson & Grapengiesser, 1999). However, the rapid transients observed during glucose stimulation usually reflects mobilization of Ca^{2+} from intracellular stores (Grapengiesser *et al.*, 1989a; Liu *et al.*, 1996). These transients are superimposed on the slow oscillations (Grapengiesser *et al.*, 1991) and mimic those obtained when the β-cells are exposed to agents promoting the formation of cAMP and IP_3 (Gylfe *et al.*, 2000). The $[Ca^{2+}]_i$ transients are synchronized in β-cells lacking physical contact, suggesting that diffusible factors, possibly nitric oxide and ATP, are involved in their generation (Grapengiesser *et al.*, 1999; Hellman *et al.*, 2001). It has been demonstrated that $[Ca^{2+}]_i$ transients, occurring together with the glucose-induced oscillations, are often sufficiently pronounced to activate a hyperpolarizing K^+ current temporarily interfering with the slow oscillations (Dryselius *et al.*, 1999). By inducing such $[Ca^{2+}]_i$ transients, nitric oxide released from nonadrenergic, noncholinergic (NANC) neurons, may entrain the slow oscillations into a rhythm common to the islets in a pancreas (Hellman *et al.*, 2000; Hellman *et al.*, 2001). In addition, ATP, coreleased with insulin into the intercellular space, may mobilize intracellular Ca^{2+} by a purinergic IP_3-mediated mechanism and provide positive feedback in both autocrine and paracrine fashion (Gylfe, 1991).

Characteristics of *ob*/*ob* mice

In mice a recessive mutant gene, *ob*, produces obesity leading to hyperinsulinemia and hyperglycemia (Ingalls *et al.*, 1950), generally referred to as the obese-hyperglycemic syndrome (Hellman, 1965). The syndrome was discovered at the Jackson Laboratory, USA in 1949 and the mice were found to be homozygous for an autosomal recessive mutation (Ingalls *et al.*, 1950). Marked obesity, hypoactivity, hyperphagia, transient hyperglycemia, severe hyperinsulinemia and insulin resistance are the cardinal features of the obese hyperglycemic syndrome when the *ob* gene is expressed in the C57BL/6J or similar strains (Westman, 1968; Dubuc, 1976; Coleman, 1978). The islets of *ob*/*ob* mice are characterized by > 90% of insulin containing β-cells with decreased proportions of glucagon-, somatostatin- and pancreatic polypeptide-containing α, δ and PP cells, respectively (Gepts *et al.*, 1960; Hellman, 1961; Baetens *et al.*, 1978). In 3-5 months old obese mice the total islet volume can be up to 8-10 times larger than in lean mice (Hellman *et al.*, 1961). The greatly increased total islet volume in the *ob*/*ob* mice is mainly dependent on an increased number of large islets (Hellman *et al.*, 1961).

In an elegant experiment, Coleman (1978) showed that parabiosis of an *ob*/*ob* mouse with a normal one suppressed weight gain in the obese mouse, whereas parabiosis with a *db*/*db* mouse caused profound weight loss and death of the *ob*/*ob* one. Taken together, these results suggest that the obese mouse does not produce sufficient satiety factor to turn off its eating drive whereas the *db*/*db* mouse lacks a functional receptor for this factor (Coleman, 1978; Friedman & Halaas, 1998). The search for the precise nature of the defect in the *ob*/*ob* mouse come to an end in 1994, when Zhang *et al*., (1994), cloned the *ob* gene and confirmed Coleman's hypothesis. The *ob* gene was isolated by positional cloning and found to encode a highly conserved 167-amino acid secretory protein, that was unique in the GenBank database (Zhang *et al.*, 1994). Mouse and human *ob* genes have been localized to chromosome 6 and 7q31.3, respectively, and the product of the *ob* gene was named leptin from the Greek word *leptos*, meaning thin, as it markedly attenuated body weight by reducing food intake and body fat when injected into *ob*/*ob* or normal mice (Zhang *et al.*,

1994; Halaas *et al.*, 1995). The recent official nomenclature for the *ob* gene in mice is *Lep*^{ob} and for the *ob*/*ob* mice on the C57BL/6J background strain is B6.V-*Lep*^{ob} (Doolittle, 1998; Jax mice web site, 2001).

Leptin is a pleotropic hormone, circulating as 16-kDa protein (Halaas *et al.*, 1995). It is synthesized and secreted primarily, but not exclusively, by white adipose tissue (Ahima & Flier, 2000). In C57BL/6J *ob*/*ob* mice, a Cys-to-Thr substitution in the *ob* gene of the chromosome 6 results in a stop codon at position 105, which produces a truncated protein, that is apparently degraded in the adipocyte (Zhang *et al.*, 1994). Thus, the *ob*/*ob* mice lack circulating leptin; and experimental studies indicate that leptin replacement corrects almost all of the abnormalities in the obese hyperglycemic syndrome (Friedman & Halaas, 1998; Ahima & Flier, 2000). Leptin mediates its intracellular signaling through binding to the specific receptor (Ob-R), which belongs to the class I cytokine receptor family (Tartaglia, 1997). Multiple splice variants of Ob-R mRNA encode at least six leptin receptor isoforms (Lee *et al.*, 1996; Ahima & Flier, 2000). However, only the long isoform, Ob-Rb contains intracellular motifs required for activation of the JAK-STAT signal transduction pathway (Bjørbæk *et al.*, 1997).

Insulin secretion in rats and mice

Glucose-induced insulin release from the isolated perfused pancreas from rats and mice is characterized by a sharp first peak followed by a second phase of sustained secretion (Lenzen, 1979). However, insulin secretion from isolated islets is much more pronounced in rats than in mice (Lenzen, 1979; Cosimi *et al.*, 1994). A rising second phase of insulin secretory response to glucose is observed in rats. In contrast, the second phase is rather flat and only minimally elevated above the basal value in mice (Berglund, 1980; Ma *et al.*, 1995). The disparity in glucose-induced insulin release in rats and mice is probably coupled to differences in cAMP and inositol phosphate accumulation in islets (Ma *et al.*, 1995; Zawalich *et al.*, 2000).

SPECIFIC AIMS

The aims of the present study were to -

1. study how the glucose-induced slow oscillations of $[Ca^{2+}]_i$ are affected by amino acids in individual mouse pancreatic β-cells and intact islets.
2. compare the Ca^{2+} handling of individual rat β-cells with that in mouse β-cells.
3. evaluate the role of the ryanodine receptors in β-cells from rats and mice.
4. analyze how the obese-hyperglycemic syndrome is reflected in the Ca^{2+} handling by individual mouse β-cells.
5. study the effects of leptin on the firing of $[Ca^{2+}]_i$ transients in β-cells from *ob*/*ob* mice.

METHODOLOGY

Animals

Experiments were performed with obese hyperglycemic mice (*ob*/*ob*), their lean heterozygous (*ob*/+) or homozygous (+/+) littermates, NMRI mice and Wistar rats. The *ob*/*ob* mouse colony was established in Sweden about 40 years ago from breeding couples obtained from Jackson Laboratory, Bar Harbor, Maine USA. The islets from these mice consist of >90% β-cells (Hellman, 1961) and are known to respond adequately to glucose and other nutrient stimuli of insulin release (Hahn *et al.*, 1974). Female NMRI and C57BL/6J mice and male Wistar rats were purchased from B&K Universal AB, Sollentuna, Sweden.

The animals were fed a standard pellet diet, R36 (Lactamin, Stockholm, Sweden) and tap water ad libitum. They were kept at 21^{o}C in 12 hours light/dark cycles. The animals were allowed to breathe CO_2 until unconsciousness and killed by decapacitation for isolation of islets. All the experiments were approved by the ethics committee for animal research at Uppsala University.

Preparation of pancreatic islets and single cells

Islets of Langerhans were isolated by collagenase digestion and either kept in culture or dissociated into single cells by shaking in a Ca^{2+}-deficient medium (Lernmark, 1974). The isolated islets were cultured for 1-4 days in RPMI 1640 medium containing 11 mM glucose and supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 30 μg/ml gentamicin. The single β-cells were suspended in identical RPMI 1640 medium. The cells were allowed to attach to circular 25 mm cover glasses and kept in culture for 1-4 days at 37^{o}C in an atmosphere of 5% CO_2 in humidified air. Unless otherwise stated subsequent experimental handling of cells was performed with a basal medium buffered with 25 mM Hepes and containing 3 mM glucose, 0.5 mg/ml bovine serum albumin, 125 mM NaCl, 4 or 5.9 mM KCl, 1.2 mM $MgCl_2$ and 1.3 mM $CaCl_2$. NaOH was used for adjusting the pH to 7.4.

Measurements of cytoplasmic Ca^{2+} in single β-cells and small aggregates

Loading with fura-2

The cells were loaded with fura-2 during 30-40 min incubation at 37°C with 0.5 μM of its acetoxymethyl ester in basal medium containing 3 mM glucose. The cover slip with the attached cells was then rinsed and mounted as bottom of an open chamber containing 160 μl medium (Sykes & Moore, 1959). The chamber wall was a broad silicon rubber ring (9 mm inner diameter) pressed to the cover slip by the threaded chamber mount and a thin stainless steel ring. Cannulas, fixed to this ring, were connected to a two-channel peristaltic pump allowing a steady superfusion of a 2.5 mm layer at a rate of 0.75 ml/min. The chamber was placed on the stage of an inverted microscope within a climate box maintained at 37°C. The microscope was equipped with an epifluorescence illuminator and a 40X or 100X UV fluorite objective.

Selection criteria for β-cells

Selection of pancreatic β-cells for analyses was based on their large size (>10 μm in diameter) and low nuclear/cytoplasmic volume ratio compared to the islet cells secreting glucagon, somatostatin and pancreatic polypeptide (Hellman, 1959; Pipeleers, 1987; Berts *et al.*, 1995; Liu *et al.*, 1999). It was checked by immunostaining that these selection criteria were appropriate in the present study.

Photomultiplier recordings

A 75 W xenon arc lamp combined with 10-13 nm half-bandwidth interference filters were used for excitation. A filter changer of a time-sharing multichannel spectrophotofluorometer provided excitation light flashes of 1 ms every 10 ms at 340 and 380 nm, respectively. To minimize the UV exposure of the cells, a quartz neutral density filter was placed between the illuminator and the filter changer. The emission was recorded at 510 nm with a photomultiplier using a 30 nm half-bandwidth filter. The electronically separated fluorescence signals

were transferred via an analog/digital converter to a personal computer using the software Genie (Advantech Co. Ltd, Taipei).

Digital image analyses

Imaging of $[Ca^{2+}]_i$ was performed with a Magiscan analysis system (VisiTech International, Sunderland, UK) using the Tardis program. Images of fura-2 loaded cells were collected at 510 nm with an intensified CCD camera after dual-wavelength excitation (Gylfe *et al.*, 1991). Pairs of 340 and 380 nm images, consisting of 16 accumulated video frames divided by 8, were captured during 2.8 sec followed by a 4 sec delay. Ratio frames were calculated after background subtraction.

Calibration of cytoplasmic Ca^{2+} measurement

$[Ca^{2+}]_i$ was calculated according to Grynkiewicz *et al.*, (1985), using the equation:

$$[Ca^{2+}]_i = K_d \cdot \frac{F_0}{F_s} \cdot \frac{R - R_{min}}{R_{max} - R}$$

where K_d is the dissociation constant of fura-2, R is the 340/380 nm fluorescence excitation ratio of fura-2, R_{max} and F_s are the 340/380 nm fluorescence excitation ratio and the 380 nm fura-2 fluorescence respectively, at saturating Ca^{2+} concentrations. R_{min} and F_0 are the corresponding values in a medium lacking Ca^{2+}. The K_d value employed was 224 nM (Grynkiewicz *et al.*, 1985). Calibration for measurement of $[Ca^{2+}]_i$ was performed in droplets of solutions mimicking the intracellular ionic milieu. R_{min} and F_0 were estimated at 37^{o}C in a buffer (pH 7.0) containing 115 mM KCl, 20 mM NaCl, 10 mM MOPS (3-[N-Morpholino]propanesulfonic acid), 1.2 mM $MgCl_2$, 5 mM EGTA (Ethylene glycol-bis[β-aminoethyl ether]-N,N,N',N'-tetraacetic acid) and 100 μM fura-2 pentapotassium salt. R_{max} and F_s were determined using a similar solution containing 10 mM $CaCl_2$ to ensure saturation of fura-2. The test substances were checked for possible interference with fura-2 measurements.

Measurements of cytoplasmic Ca^{2+} in intact islets

The islets were loaded with fura-2 during 45 min incubation at 37°C with 2 μM of its acetoxymethyl ester together with 0.02% (w/v) Pluronic F-127. After loading the islets were allowed to attach to cover glasses coated with poly-L-lysine and $[Ca^{2+}]_i$ was measured with a photomultiplier (see above). The analyses were restricted to islets responding to 11 mM glucose with slow oscillations of $[Ca^{2+}]_i$. Measurements were made in an optical plane close to the lower surface of the islets. The fluorescence excitation ratio remained unaffected during glucose stimulation of islets lacking the fura-2 indicator. The $[Ca^{2+}]_i$ values have therefore been given without compensation for autofluorescence, which was <15%.

Statistical evaluation

Results are presented as mean values ± standard error of means. In the photomultiplier recordings each experiment refers to analyses of individual β-cells or islets on separate cover slips. In the image analyses, unless otherwise stated, each experiment refers to the average number of transients (> 50 nM) found in 4-12 cells from the same cover slip. Transients occurring within 3 successive ratio frames were considered synchronized. A given protocol was tested with cells from at least 5 animals. Statistical analyses were performed with Student's *t*-test and chi-square test with Yates' correction.

RESULTS AND DISCUSSION

Effects of amino acids on glucose-induced slow $[Ca^{2+}]_i$ oscillations (I)

The periodic variations of circulating insulin due to pulsatile release of the hormone are explained by the $[Ca^{2+}]_i$ oscillations in the pancreatic β-cells. It was therefore investigated how the slow $[Ca^{2+}]_i$ oscillations are affected by various amino acids. Individual *ob*/*ob* and NMRI mouse β-cells with glucose-induced slow $[Ca^{2+}]_i$ oscillations were exposed to glycine, alanine and arginine at concentrations close to the physiological range. Each of these amino acids transformed the oscillations into sustained elevation of $[Ca^{2+}]_i$ when added at concentrations as low as 0.1 or 0.5 mM. However, in intact pancreatic islets much higher concentrations of the amino acids were required to transform the slow $[Ca^{2+}]_i$ oscillations into sustained elevation. Even at the highest amino acid concentration tested (10 mM), the glucose-induced $[Ca^{2+}]_i$ oscillations continued in about 45% of the islets.

Role of electrogenic transport

The sustained elevation of $[Ca^{2+}]_i$ in response to glycine and alanine is probably due to the depolarizing effect of the Na^+, cotransported with the amino acids via the transport systems A, ASC and GLY (Christensen, 1990). It is evident from electrophysiological studies that alanine has a depolarizing action on primary (Henquin & Meissner, 1981) as well as clonal β-cells (Dunne *et al.*, 1990). The failure of glycine to influence $[Ca^{2+}]_i$ and insulin release (Tengholm *et al.*, 1992), and of alanine to modulate electrical activity and $[Ca^{2+}]_i$ (Henquin & Meissner, 1986; Dunne *et al.*, 1990) in the absence of Na^+ indicates that influx of this cation is a major component in the mechanism of action of the amino acids in pancreatic β-cells. Indeed, Na^+ entry in response to veratridine triggers transients of $[Ca^{2+}]_i$ due to opening of the voltage-dependent Ca^{2+} channels (Eberhardson & Grapengiesser, 1999). An increase of the cytoplasmic Na^+ concentration can also elevate $[Ca^{2+}]_i$ by mobilizing Ca^{2+} from intracellular stores and inhibiting the outward transport of Ca^{2+} by the Na^+/Ca^{2+} exchanger (Hellman *et al.*, 1982; Charles & Henquin, 1983).

The glucose-induced oscillations of $[Ca^{2+}]_i$ were transformed into sustained elevation by the positively charged amino acid, arginine. There is convincing evidence that the electrogenic transport of arginine by the murine cationic amino acid transporter accounts for the depolarization, which results in the increase of $[Ca^{2+}]_i$ and potentiation of insulin release (Blachier *et al.*, 1989; Smith *et al.*, 1997; Sener *et al.*, 2000). In a transgenic β-cell line, NIT-1, arginine-induced increase in $[Ca^{2+}]_i$ was found to be Na^+-independent but required the influx of Ca^{2+} (Weinhaus *et al.*, 1997). It seems unlikely that generation of nitric oxide or metabolites of arginine contribute to the $[Ca^{2+}]_i$-elevating effect of arginine in pancreatic β-cells (Yada, 1994; Smith *et al.*, 1997).

Role of Ca^{2+} permeability

Previous studies have revealed that the glucose-induced slow oscillations of $[Ca^{2+}]_i$ are critically dependent on the rate of Ca^{2+} entry (Eberhardson *et al.*, 1996). The present study supports such a role demonstrating that both a rise of extracellular Ca^{2+} (10 mM) or addition of the Ca^{2+} channel agonist BAY K8644 re-establishes the slow $[Ca^{2+}]_i$ oscillations suppressed by the amino acids. Enhanced Ca^{2+} influx through VDCC may trigger a temporary rise of $[Ca^{2+}]_i$, which overshoots the $[Ca^{2+}]_i$ level in the presence of amino acids. This rise of $[Ca^{2+}]_i$ could activate an outward hyperpolarizing K^+ current (Dryselius *et al.*, 1999) and/or inhibit the inward Ca^{2+} current by direct inhibition of the voltage-dependent channels (Rorsman & Trube, 1986), thereby terminating the influx of Ca^{2+}. However, since the $[Ca^{2+}]_i$ rhythmicity seems to be determined by oscillations in metabolism (Dryselius *et al.*, 1994), it can be anticipated that increased influx of Ca^{2+} somehow restores the influence of metabolism on $[Ca^{2+}]_i$ oscillations. Such an effect could result from the increase of $[Ca^{2+}]_i$ in the submembrane space causing an enhanced consumption of ATP for removal of the ion by the Ca^{2+}ATPase and the Na^+/Ca^{2+} exchanger. The subsequent lowering of the ATP/ADP ratio may be sufficient to open K^+_{ATP} channels and induce repolarization (Grapengiesser, 1998).

Role of islet cell interactions

The slow $[Ca^{2+}]_i$ oscillations in β-cells situated in islets were more resistant to transformation into sustained elevation of $[Ca^{2+}]_i$ by the amino acids. The $[Ca^{2+}]_i$ oscillations in a syncytium of islet cells are determined not only by the individual β-cells but also by synchronizing signals from adjacent β-cells and structural and functional coupling among β-cells and glucagon producing α-cells (Gylfe *et al.*, 1991; Hellman *et al.*, 1994; Grapengiesser *et al.*, 1999; Göpel *et al.*, 1999). It is likely that these interactions make it possible for the β-cells to overcome the suppression of the oscillatory activity otherwise obtained in the presence of low concentrations of glycine, alanine or arginine.

Glucose induction of slow $[Ca^{2+}]_i$ oscillations and rapid transients in rat and mouse β-cells (II, III)

With the development of sensitive fluorescent Ca^{2+}-indicators it became possible to demonstrate that the glucose-induced increase of $[Ca^{2+}]_i$ in pancreatic β-cells is usually manifested as well-shaped slow oscillations in $[Ca^{2+}]_i$ (Grapengiesser *et al.*, 1988; Liu *et al.*, 1998). In mouse pancreatic β-cells, the pattern of glucose-induced oscillatory $[Ca^{2+}]_i$ changes appears consistent in different studies (Hellman *et al.*, 1992; Jonkers *et al.*, 1999). However, in rat β-cells heterogeneous responses to glucose have been reported (Wang & McDaniel, 1990; Pralong *et al.*, 1990; Herchuelz *et al.*, 1991; Theler *et al.*, 1992; Yada *et al.*, 1992). The differences in $[Ca^{2+}]_i$ responses between rats and mice may be related to technical factors. Rat islets seem more sensitive to manipulation during isolation and the β-cells do not readily attach to cover glasses necessitating the use of poly-L-lysine, which may have some toxic effect. A systematic improvement of the technique with gentle handling of cells and minimizing the exposure to UV-light during the measurements made it possible to increase the percentage of both mouse and rat β-cells responding to glucose with $[Ca^{2+}]_i$ oscillations as high as 80%.

Under basal conditions $[Ca^{2+}]_i$ usually remained stable at 60-90 nM when single rat pancreatic β-cells were exposed to 3 mM glucose. After raising the glucose concentration to 11 mM there was an initial decrease in $[Ca^{2+}]_i$ by 21%

followed by a sharp rise with a latency period of 2.38 ± 0.32 min (n = 44). The initial peak was in most cases more pronounced than subsequent oscillations in $[Ca^{2+}]_i$. The oscillations in the rat β-cells originated from the $[Ca^{2+}]_i$ level of about 107 nM with a frequency of about 0.23/min and amplitude of about 343 nM (n = 44). Occasionally the exposure to 11 mM glucose caused a monophasic increase in $[Ca^{2+}]_i$ characterized by a sharp rise followed by a return to near basal levels or to a sustained elevation, sometimes with superimposed rapid transients. Stimulation of single pancreatic β-cells from mice resulted in similar response patterns. However, the $[Ca^{2+}]_i$ oscillations in mouse β-cells had a slightly higher frequency (Table 1).

The glucose-induced oscillations were sometimes superimposed with transients of $[Ca^{2+}]_i$. Blocking the voltage-dependent Ca^{2+} entry with methoxyverapamil made it possible to study the transients without disturbance from the slow oscillations of $[Ca^{2+}]_i$ (Liu *et al.*, 1996). Using this approach, both in rats and mice, it was found that the $[Ca^{2+}]_i$ transients were coordinated in time even in β-cells lacking physical contact. The frequency of $[Ca^{2+}]_i$ transients in rat β-cells tended to be higher than seen under similar conditions in mouse β-cells.

Table 1. Characteristics of glucose-induced slow $[Ca^{2+}]_i$ oscillations in single β-cells from rats (Wistar) and mice (C57BL/6J).

Characteristics	Rat β-cells	Mouse β-cells	*P* value
Frequency (oscill/min)	0.23 ± 0.01	0.31 ± 0.02	< 0.001
Amplitude (nm)	343 ± 14	349 ± 26	> 0.05
Half-width (min)	1.60 ± 0.10	1.27 ± 0.10	> 0.05

The oscillations were induced by 11 mM glucose in individual pancreatic β-cells. Data are presented as mean values ± SEM.

Glucagon modulation of glucose-induced slow $[Ca^{2+}]_i$ oscillations in rat and mouse β-cells (II, III)

In pancreatic β-cells, glucagon binds with high affinity to its receptors (Moens *et al.*, 1996; Huypens *et al.*, 2000) and raises the cAMP concentrations, which modulate both the β-cell handling of Ca^{2+} and its effects on insulin release. Cyclic AMP exerts multiple effects on pancreatic β-cells, such as increase of Ca^{2+} influx through VDCC (Ämmälä *et al.*, 1993; Kanno *et al.*, 1998), mobilization of Ca^{2+} from intracellular stores (Holz *et al.*, 1995; Liu *et al.*, 1996), increased Ca^{2+} sensitivity of the secretory machinery (Ämmälä *et al.*, 1993; Renström *et al.*, 1997) as well as modulation of K_{ATP} (Yaekura *et al.*, 1996; Gromada *et al.*, 1997) and nonselective cation channels (Holz *et al.*, 1995). In the present study, the addition of 10 nM glucagon usually resulted in a rapid transformation of the oscillations into a sustained elevation. It is noteworthy that the sustained elevation of $[Ca^{2+}]_i$ in rat β-cells was preceded by a temporary lowering. However, in mouse β-cells the oscillations often persisted in the presence of glucagon. The complexity of the cAMP action in rat β-cells may be due to dual effects of the nucleotide on $[Ca^{2+}]_i$ in stimulating both the entry of Ca^{2+} and its removal from the cytoplasm (Yaekura *et al.*, 1996; Yaekura & Yada, 1998). The finding that the oscillations were more readily transformed into sustained elevation of $[Ca^{2+}]_i$ in rat than in mouse β-cells is consistent with a reported less negative resting membrane potential compared with mouse β-cells (Antunes *et al.*, 2000). It is also likely that rat β-cells have a greater production of, and sensitivity to, cAMP (Ma *et al.*, 1995). Thams *et al.*, (1988) have proposed that both glucose and carbachol potentiate cAMP formation in the presence of endogenous glucagon in mouse islets. It has also been demonstrated that the compounds that elevate cAMP concentrations, and initiate PLC activity, act synergistically on the insulin secretory process both in rat and mouse islets (Zawalich, 1988; Zawalich & Zawalich, 2001). Thus, the differential modulatory action of glucagon on $[Ca^{2+}]_i$ oscillations in rat and mouse β-cells might also reflect differences in glucose-stimulated PLC-mediated inositol phosphate accumulation (Zawalich *et al.*, 2001).

Glucagon modulation of glucose-induced $[Ca^{2+}]_i$ transients (III)

Glucose stimulation of mouse β-cells is known to involve generation of brief transients of $[Ca^{2+}]_i$, a phenomenon particularly obvious when the IP_3 receptors are sensitized by cAMP (Liu *et al.*, 1996). In rat pancreatic β-cells glucagon lacked effect on $[Ca^{2+}]_i$ when added to a K^+-rich medium containing 3 mM glucose. However, in a similar medium containing 20 mM glucose addition of glucagon produced $[Ca^{2+}]_i$ transients with a frequency of 0.50 ± 0.11/min (n=17) in 57% of the cells. The observation that the generation of $[Ca^{2+}]_i$ transients is glucose-dependent corroborates previous data in mouse β-cells (Liu *et al.*, 1996). There are reports indicating permissive effects of glucose on glucagon-induced increase of $[Ca^{2+}]_i$ (Grapengiesser *et al.*, 1991), cAMP formation (Schuit & Pipeleers, 1985), and insulin release (Schauder *et al.*, 1977). Moreover, IP_3-induced Ca^{2+} mobilization is dependent on the glucose concentration (Gylfe, 1991), and it has recently been shown that the Ca^{2+} accumulation in the ER is maximally stimulated by 20 mM glucose, an effect mediated by a rise of ATP (Tengholm *et al.*, 1999). Elevation of $[Ca^{2+}]_i$ by K^+ depolarization accelerates the ER sequestration of Ca^{2+} in response to glucose (Tengholm *et al.*, 2001). In the present study, the frequency of transients was higher in β-cells depolarized with K^+ than in those where $[Ca^{2+}]_i$ was kept low by blocking the voltage-dependent entry of the ion with methoxyverapamil. The observation that rise of $[Ca^{2+}]_i$ stimulates the firing of transients is coherent with the reports that type 3 is the predominant subtype of the IP_3 receptors in rat β-cells (Blondel *et al.*, 1993; Lee *et al.*, 1998) and that this isoform of the IP_3 receptors provides a positive feedback on Ca^{2+} mobilization with rise of $[Ca^{2+}]_i$ (Hagar *et al.*, 1998). Depolarization *per se* can increase the production of IP_3 in β-cells independent of Ca^{2+} influx (Liu *et al.*, 1996) and it is also known that cAMP sensitizes the IP_3 receptors by phosphorylation (Nakade *et al.*, 1994; LeBeau *et al.*, 1999). Such a crosstalk between the cAMP and IP_3 signaling pathway, the effects of glucose on Ca^{2+} sequestration in the ER and of $[Ca^{2+}]_i$ on the IP_3 receptors are important determinants for the generation of $[Ca^{2+}]_i$ transients in pancreatic β-cells.

To study the contribution of the intracellular Ca^{2+} stores in the generation of transients, thapsigargin was used. This compound specifically inhibits the SERCA pump, causing a rapid Ca^{2+} depletion of the ER (Thastrup *et al.*, 1990; Inesi & Sagara, 1994). Since the IP_3 receptors are preferentially localized in the ER (Blondel *et al.*, 1993; Hagar *et al.*, 1998), it was not surprising that the transients disappeared after addition of thapsigargin to rat β-cells. Although low concentrations of caffeine act as phosphodiesterase inhibitor and mimic the effect of glucagon in raising cAMP and inducing $[Ca^{2+}]_i$ transients (Liu *et al.*, 1996), the IP_3 receptors from various cell types are inhibited by high concentrations of the drug. Caffeine, at high concentrations, competes for the ATP binding sites of both IP_3R1 and IP_3R3 (Maes *et al.*, 2000) and inhibits agonist-induced formation of IP_3 (Toescu *et al.*, 1992; Combettes *et al.*, 1994). This compound is known to interfere with IP_3-mediated mobilization of Ca^{2+} also in mouse β-cells (Lund & Gylfe, 1994; Liu *et al.*, 1996). When added at high concentrations, caffeine increases the mean open time of the ryanodine receptor channels (Shoshan-Barmatz & Ashley, 1998) and enhances their sensitivity to Ca^{2+} (Sitsapesan & Williams, 1997). The present finding that, 20 mM caffeine abolishes the $[Ca^{2+}]_i$ transients in individual rat β-cells suggests that the IP_3 receptor is primarily responsible for glucose-induced generation of these transients.

Role of ryanodine receptors for glucose induction of slow $[Ca^{2+}]_i$ oscillations (II, III)

Both caffeine and ryanodine act on the Ca^{2+}-induced Ca^{2+} release mechanism in different cell types (Endo, 1977; Iino, 1989; Friel & Tsien, 1992) including pancreatic β-cells (Lemmens *et al.*, 2001). Although multiple effects have been attributed to caffeine (Tung *et al.*, 1990; Lund & Gylfe, 1994; Islam *et al.*, 1995; Sei *et al.*, 2001), this compound has been used extensively for studying ryanodine receptors (Shoshan-Barmatz & Ashley, 1998). There are several reports that caffeine mobilizes Ca^{2+} from intracellular stores in pancreatic β-cells from rats (Willmott *et al.*, 1995), *ob*/*ob* mice (Islam *et al.*, 1998), RINm5F (Chen *et al.*, 1996), HIT-T15 cells (Li *et al.*, 1996) and INS β-cell lines (Gamberucci *et al.*, 1999). However, depending on the concentration,

caffeine can either stimulate or inhibit intracellular Ca^{2+} release (Wakui *et al.*, 1990; Liu *et al.*, 1996). Caffeine has been reported to inhibit the Ca^{2+} oscillations induced by agonists in different cell types, including *Xenopus* oocytes (Parker & Ivorra, 1991), pancreatic acinar cells (Sjödin & Gylfe, 2000) and isolated hepatocytes (Combettes *et al.*, 1994). In the present study caffeine negatively modulated the slow $[Ca^{2+}]_i$ oscillatory activity in a dose-dependent and reversible manner. The glucose-induced oscillations in rat pancreatic β-cells persisted in the presence of 2 mM and 10 mM caffeine although their amplitudes were sometimes attenuated by 10 mM caffeine. Increase of the caffeine concentration to 20 mM resulted in a disappearance of the oscillations with sustained elevation of $[Ca^{2+}]_i$ at 20 ± 3 nM above the oscillatory nadirs. Similar results were obtained when caffeine was added in different concentrations to individual mouse (C57BL/6J) β-cells. These findings are consistent with the report that caffeine has direct effects on the Ca^{2+} entry into the β-cells additional to those mediated by an increase of cAMP (Islam *et al.*, 1995; Li *et al.*, 1996) or related to the IP_3-mediated Ca^{2+} signaling (Lund & Gylfe, 1994; Li *et al.*, 1996). Direct effects of caffeine on Ca^{2+} influx has been demonstrated in many other cell types like lymphocytes (Sei *et al.*, 2001), clonal rat pituitary cells (Karhapää & Törnquist, 1997), hepatocytes (Combettes *et al.*, 1994), vascular smooth muscle cell line (Otun *et al.*, 1991), and cardiac (Zahradnik & Palade, 1993) and smooth muscle cells (Guerrero *et al.*, 1994). In the pancreatic β-cells caffeine lowered the $[Ca^{2+}]_i$ also in a K^+-rich medium containing 3 or 20 mM glucose, supporting the idea that the effects of caffeine is due to suppression of the voltage-dependent Ca^{2+} entry rather than interference with glucose metabolism.

The plant alkaloid ryanodine binds to the Ca^{2+} release channels in pancreatic β-cells from rats, mice and humans as well as in clonal β-cell lines (Islam *et al.*, 1998; Holz *et al.*, 1999). Different opinions have been expressed regarding the functional importance of ryanodine receptors for mobilizing Ca^{2+} from intracellular stores in pancreatic β-cells (Takasawa *et al.*, 1993; Rutter *et al.*, 1994; Islam *et al.*, 1998; Holz *et al.*, 1999; Tengholm *et al.*, 2000). When rat and mouse β-cells were exposed to 5-20 μM ryanodine, the glucose-induced slow $[Ca^{2+}]_i$ oscillations remained unaffected. However, the addition of

ryanodine sometimes re-established oscillations inhibited by caffeine. The current finding, that ryanodine reverses the effects of caffeine, is consistent with the idea that ryanodine acts only on the open state of the Ca^{2+} release channel (Iino *et al.*, 1988; Teraoka *et al.*, 1991), locking it in an open sub-conductance state (Buck *et al.*, 1992; Cheek *et al.*, 1993). The present data therefore provide some evidence for conditional modulation of ryanodine receptors in β-cells.

β-cell handling of Ca^{2+} in obese-hyperglycemic mice (I, II)

Obese-hyperglycemic mice have been extensively used in the studies of β-cell metabolism and the stimulus-secretion coupling mechanism, including the regulation of $[Ca^{2+}]_i$ (Hellman, 1970; Hellman & Gylfe, 1986). These mice are characterized by especially large and numerous pancreatic islets (Hellman *et al.*, 1961; Leckström *et al.*, 1999) with a high proportion of β-cells (Hellman, 1961). It has been proposed that the increased number of β-cells represents the normal adaptation to hyperglycemia (Hellman, 1970). The manifestation of the obese-hyperglycemic syndrome depends critically on background strain, age, diet and nutritional state (Coleman, 1978; Flatt *et al.*, 1992). The Swedish colony of non-inbred *ob*/*ob* mice have a similar syndrome as that observed with the *ob* gene on the inbred C57BL/6J strain (Westman, 1968; Coleman, 1978). The islets from the Swedish *ob*/*ob* mice have been reported to respond adequately to various stimulators and inhibitors of insulin release, including glucose (Lernmark & Hellman, 1969; Hahn *et al.*, 1974). It has also been demonstrated that a regular pattern of pulsatile insulin release is generated by isolated *ob*/*ob* islets in response to slow oscillations of $[Ca^{2+}]_i$ (Bergsten *et al.*, 1994).

In the present study, the effects of glucose and amino acids on $[Ca^{2+}]_i$ were tested in single β-cells from *ob*/*ob* and lean mice. There were no differences in the frequency, amplitude and half-width of the glucose-induced oscillations in the two types of mice. However, after addition of 10 mM caffeine, the slow $[Ca^{2+}]_i$ oscillations were usually transformed into sustained increase of $[Ca^{2+}]_i$ in β-cells from the obese but not from lean mice. The observation that 10 mM caffeine transforms the oscillations into sustained elevation in *ob*/*ob* mice is

consistent with the report that caffeine has effects on the Ca^{2+} entry into the β-cells additional to those mediated by an increase of cAMP (Islam *et al.*, 1995).

The transients of $[Ca^{2+}]_i$, sometimes observed to be superimposed on the slow oscillations when the β-cells were stimulated with glucose, became more pronounced in the presence of glucagon. Since the cAMP concentrations in β-cells within intact islets are elevated by endogenously released glucagon (Schuit & Pipeleers, 1985), such transients are probably representative for the physiological situation. In a glucagon-containing medium, glucose was more effective in triggering $[Ca^{2+}]_i$ transients in β-cells from *ob*/*ob* than in those from lean mice. Also after blocking the Ca^{2+} entry with methoxyverapamil it was found that the generation of $[Ca^{2+}]_i$ transients was higher in β-cells from *ob*/*ob* mice both in control situation and in the presence of glucagon, caffeine, carbachol or ryanodine. It is possible that the excessive firing of $[Ca^{2+}]_i$ transients in the *ob*/*ob* mouse β-cells is related to an increased activity of PLC (Zawalich & Zawalich, 1996; Chen & Romsos, 1997) and a hypersensitivity to cAMP (Black *et al.*, 1986; Fournier *et al.*, 1994).

Effects of leptin on glucose-induced $[Ca^{2+}]_i$ transients (II)

The pancreatic β-cells express several isoforms of the leptin receptor, including the full-length receptor Ob-Rb (Kieffer *et al.*, 1996; Emilsson *et al.*, 1997), implying that leptin can modulate β-cell handling of Ca^{2+} (Fehmann *et al.*, 1997) and insulin secretion (Kieffer & Habener, 2000). The exaggerated insulin secretory response to muscarinic receptor agonists in *ob*/*ob* mice is thought to be mediated by a phopholipase C-activated pathway, which is suppressed by leptin in lean mice (Zawalich & Zawalich, 1996; Chen & Romsos, 1997). The present study provides additional arguments for a direct interaction of leptin with pancreatic β-cells, in demonstrating that this hormone suppresses the firing of $[Ca^{2+}]_i$ transients. The leptin effect was evident already at 1 nM, a concentration close to that in circulating blood of normal rodents (Poitout *et al.*, 1998). The leptin suppression of the $[Ca^{2+}]_i$ transients may not only be due to a decreased PLC activity but also to an interference with cAMP sensitization of the IP_3 receptor. Leptin has been reported to reduce the cAMP concentration

due to an increased phosphodiesterase 3B activity both in an insulinoma cell line and rat pancreatic β-cells (Zhao *et al.*, 1998). Whereas leptin inhibits cAMP-induced release of insulin without affecting the secretory response to PLC activation in insulinoma cells (Ahrén & Havel, 1999), it has been reported to constrain the PLC-mediated insulin secretion from *ob*/*ob* mouse islets (Chen *et al.*, 1997).

CONCLUSIONS

1. Glucose-induced slow oscillations of $[Ca^{2+}]_i$ in isolated mouse β-cells are transformed into sustained elevation by glycine, alanine and arginine at concentrations as low as 0.1 mM. After stimulating Ca^{2+} entry, the oscillatory activity often reappears in β-cells exposed to the amino acids. The slow $[Ca^{2+}]_i$ oscillations are more resistant to amino acid transformation into the sustained elevation in intact islets than in isolated β-cells, supporting the idea that islet cell interactions are important for maintaining the oscillatory activity.

2. Individual rat β-cells respond to glucose stimulation with slow $[Ca^{2+}]_i$ oscillations, due to periodic entry of Ca^{2+}, as well as with transients evoked by mobilization of intracellular stores. The $[Ca^{2+}]_i$ oscillations in rat β-cells have a slightly lower frequency than those in mouse β-cells and are more easily transformed into sustained elevation. The $[Ca^{2+}]_i$ transients are more frequent in rat than in mouse β-cells and often appear in synchrony also in cells lacking direct physical contact.

3. In accordance with the idea that β-cells have functionally active ryanodine receptors, ryanodine sometimes restores oscillatory activity abolished by caffeine. However, there is little doubt that the IP_3 receptor is the major Ca^{2+} release channels both in mouse and rat β-cells.

4. Single β-cells from *ob*/*ob* mice do not differ from those of lean controls with regard to frequency, amplitudes and half-widths of the slow $[Ca^{2+}]_i$ oscillations. Nevertheless, there is an excessive firing of $[Ca^{2+}]_i$ transients in the β-cells from the *ob*/*ob* mice.

5. Leptin at a concentration as low as 1 nM suppresses the firing of $[Ca^{2+}]_i$ transients in β-cells from *ob*/*ob* mice. The excessive β-cell firing of $[Ca^{2+}]_i$ transients in *ob*/*ob* mice may be due to absence of leptin and mediated by activation of the phospholipase C signaling pathway.

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