EXPERIMENTAL STUDY

Glucose-regulated pulsatile insulin release from mouse islets via the K_{ATP} channel-independent pathway

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Abstract

Objective: Regulation of insulin release by glucose involves dual pathways, including or not inhibition of ATP-sensitive K^+ channels (K_{ATP} channels). Whereas the K_{ATP} channel-dependent pathway produces pulsatile release of insulin it is not clear whether the independent pathway also generates such kinetics.

Design and methods: To clarify this matter, insulin secretion and cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) were studied in perfused pancreatic islets from *ob/ob* mice. Insulin release was measured by ELISA technique and $[Ca^{2+}]_i$ by dual-wavelength fluorometry.

Results: Insulin secretion was pulsatile (0.2–0.3/min) at 3 mmol/l glucose when $[Ca^{2+}]_i$ was low and stable. Stimulation with 11 mmol/l of the sugar increased the amplitude of the insulin pulses with maintained frequency and induced oscillations in $[Ca^{2+}]_i$. Permanent opening of the K_{ATP} channels with diazoxide inhibited glucose-stimulated insulin secretion back to basal levels with maintained pulsatility despite stable and basal $[Ca^{2+}]_i$ levels. Increase of the K^+ concentration to 30.9 mmol/l in the continued presence of diazoxide and 11 mmol/l glucose restored the secretory rate with maintained pulsatility and caused stable elevation in $[Ca^{2+}]_i$. Simultaneous introduction of diazoxide and elevation of K^+ augmented average insulin release almost 30-fold in 3 mmol/l glucose with maintained pulse frequency. Subsequent elevation of the glucose concentration to 11 and 20 mmol/l increased the release levels. After prolonged exposure to diazoxide, elevated K^+ and 20 mmol/l glucose, the pulse frequency decreased significantly.

Conclusions: Not only glucose signaling via the K_{ATP} channel-dependent but also that via the independent pathway generates amplitude-modulated pulsatile release of insulin from isolated islets.

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Introduction

Ever since the discovery of regular fluctuations of blood insulin levels in normal subjects (1), and the disturbance of this pattern in diabetes (2), attempts have been made to understand the nature of these fluctuations. The plasma insulin oscillations are produced by intermittent release of the hormone from the pancreas (3), which requires coordination of pulsatile secretion from the individual islets of Langerhans (4). Changes in the glucose concentration modulate the amplitude of the circulating insulin oscillations (5) as well as the pulsatile secretion (4) without affecting the frequency. Insulin secretion is regulated by the ambient glucose concentration, which is sensed by the metabolism of the sugar in the pancreatic β -cell (6). Glucose metabolism augments the production of ATP, raising the ATP/ADP ratio and reducing the permeability of the ATP-sensitive K^+ channels (K_{ATP} channels). The resulting depolarization and opening

of voltage-dependent L-type Ca^{2+} channels causes influx of Ca^{2+} and elevation of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$), the most important trigger of exocytosis of insulin granules (7). According to this K_{ATP} channel-dependent mechanism variations in the ATP/ADP ratio are generally believed to generate pulsatile insulin release (8). However, opinions differ whether the variations in ATP/ADP ratio occur as a consequence of primary oscillations in metabolism (9–11) or are due to the fluctuations in the energy-requiring removal of Ca^{2+} from the cytoplasm (12). It follows from these concepts that oscillations of $[Ca^{2+}]_i$ occur in parallel with pulsatile insulin release, and such a correlation has been repeatedly demonstrated under both physiological (13, 14) and non-physiological (15) conditions.

The K_{ATP} channels apparently play an important role in glucose-stimulated insulin secretion, linking metabolic and ionic events in the β -cell (16), generating pulsatile insulin release (17). Glucose also has a

stimulatory effect on insulin secretion independent of the K_{ATP} channel. This pathway can be studied by keeping the K_{ATP} channel permanently open with diazoxide and depolarizing the β -cells with elevated concentrations of K^+ (18–22). Under such conditions, $[Ca^{2+}]_i$ is raised but remains stable without oscillations (18, 19). The K_{ATP} channel-independent glucose-signaling pathway has been demonstrated also in human islets (23). Given the close relationship between $[Ca^{2+}]_i$ and insulin release (13–15), it was not surprising that insulin secretion from individual islets was reported to be non-pulsatile under such conditions (24). However, in another study pulsatile insulin release was observed when batches of islets were perfused with diazoxide and elevated K^+ (20). Moreover, several conditions have been reported when insulin release remains pulsatile when $[Ca^{2+}]_i$ is stable, including exposing islets to diazoxide alone (25), high concentrations of glucose (26), tolbutamide or depolarizing them with elevated K^+ (27). We have now analyzed the kinetics of insulin secretion from individual *ob/ob* mouse islets, whose large size and high content of β -cells (28) made such measurements feasible. It will be shown that glucose-stimulated insulin release remains pulsatile and amplitude-regulated also when the K_{ATP} channels are permanently opened with diazoxide and the membrane potential is clamped at depolarized levels with elevated concentrations of K^+ .

Materials and methods

Materials

Reagents of analytical grade and deionized water were used. Collagenase, Hepes and BSA (fraction V) were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Tetramethylbenzidine and insulin-peroxidase came from Sigma (St Louis, MO, USA). The rat insulin standard was from Novo Nordisk (Bagsvaerd, Denmark). IgG-certified microtiter plates were purchased from Nunc (Roskilde, Denmark). Diazoxide was a gift from Schering Corp. (Kenilworth, NJ, USA). The mouse insulin antibodies were raised in our laboratory in guinea pigs.

Preparation and culture of islets

Pancreatic islets were collagenase isolated from *ob/ob* mice taken from a local colony (28). Freshly isolated islets were used for all insulin experiments, which were performed in a medium supplemented with 1 mg/ml albumin and containing (in mmol/l): NaCl 125, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1.3 and Hepes 25, titrated to pH 7.4 with NaOH. When KCl was increased to 30.9 mmol/l, NaCl was isoosmotically reduced. For the $[Ca^{2+}]_i$ experiments, the islets were cultured overnight in the presence of 5.5 mmol/l glucose in RPMI

1640 supplemented with 10% fetal calf serum. The Ca^{2+} concentration of the perfusion medium was adjusted to 2.6 mmol/l $CaCl_2$. Islets from different animals were used for each experiment.

Measurements of insulin release

The kinetics of insulin release were studied essentially as described previously (4). A single islet was placed in a thermostated (37 °C) 10 μ l chamber and perfused at a constant flow rate with a medium containing 3 mmol/l glucose. The flow rate was kept constant throughout each experiment with the aid of a peristaltic pump placed before the islet. Flow variation of 150–200 μ l/min was allowed between experiments. After 60–75 min of introductory perfusion the perfusate was collected in 20 s fractions directly into microtiter plates. Insulin was assayed by a competitive ELISA with the insulin antibody immobilized directly onto the solid phase. Amounts of insulin down to 100 amol were obtained from linear standard curves in semilogarithmic plots. The rate of insulin release was normalized to dry weight after freeze-drying and weighing the islets on a quartz fiber balance. In order to establish the variations in the assay under the present experimental conditions, perfusions were performed with known amounts of insulin without islets in the perfusion chamber. No regular variations in insulin determinations were detected.

Measurement of $[Ca^{2+}]_i$

Individual islets were loaded with 2 μ mol/l fura 2-acetoxymethyl ester in the presence of 3 mmol/l glucose for 50 min. Each islet was attached to a poly-L-lysine-coated coverslip serving as bottom of a 150 μ l perfusion chamber. The chamber was placed on the stage of an inverted microscope (Diaphot; Nikon Inc, Melville, NY, USA) within a climate box maintained at 37 °C. $[Ca^{2+}]_i$ was recorded with dual-wavelength fluorometry with excitation at 340 and 380 nm and emission at 510 nm and calculated as previously described (29).

Data analysis

Frequency determination of insulin pulses and $[Ca^{2+}]_i$ oscillations was done by Fourier transformation using the Igor software (Wave Metrics Inc., Lake Oswego, OR, USA). Determination of significant insulin pulses was based on the signal-to-noise ratio as described previously (14).

Statistical analysis

Results are presented as means $\pm$ S.E.M. Differences in secretory rates and frequencies were evaluated with

Student's two-tailed *t*-test for paired and unpaired observations.

Results

In the presence of 3 mmol/l glucose, basal insulin release was modest, corresponding to 11 ± 2 pmol/g per s and displaying pulses with a frequency of about 0.3/min (Table 1). In separate experiments, $[Ca^{2+}]_i$ was low and stable around 100 nmol/l under these conditions (Fig. 1b). Increase of the glucose concentration to 11 mmol/l caused an almost 20-fold increase in the average insulin secretion, largely due to enhanced amplitude of the insulin pulses without any change in their frequency (Fig. 1a, Table 1). When measuring $[Ca^{2+}]_i$, an increase of glucose to 11 mmol/l resulted in

Table 1 Insulin release from isolated islets after sequential introduction of diazoxide and elevated K^+ . Individual pancreatic islets were perfused in the presence of 3 and 11 mmol/l glucose. Diazoxide was added to the perfusion medium before the extracellular K^+ concentration was elevated. Values are means $\pm$ S.E.M. for six experiments.

Glucose (mmol/l)	Diazoxide (μ mol/l)	K^+ (mmol/l)	Insulin release	
			pmol $\cdot$ g $^{-1}$ $\cdot$ s $^{-1}$	Oscillations (per min)
3	—	5.9	11 ± 2	0.32 ± 0.09
11	—	5.9	$216 \pm 74^*$	0.25 ± 0.07
11	400	5.9	$12 \pm 4^*$	0.22 ± 0.02
11	400	30.9	$254 \pm 91^*$	0.26 ± 0.03

* *P* < 0.001 versus preceding perfusion condition.

Figure 1 Glucose-induced insulin release (a) and changes in $[Ca^{2+}]_i$ (b) of individual islets after introduction of diazoxide and elevated K^+ . Individual mouse islets were perfused in the presence of 3 and 11 mmol/l glucose. The open arrow indicates when 400 μ mol/l diazoxide were added to the perfusion medium and the closed arrow when the K^+ concentration of the medium was increased to 30.9 mmol/l. Representative experiments of *n* = 6 (a) and *n* = 3 (b).

Figure 2 Glucose-induced insulin release from individual islets after introduction of diazoxide. Individual mouse islets were perfused in the presence of 3 and 11 mmol/l glucose. The arrow indicates when 400 μ mol/l diazoxide were added to the perfusion medium. Representative experiment of $n = 4$.

initial lowering followed by a pronounced peak and subsequent oscillations with the major frequency component corresponding to $0.56 \pm 0.15/\text{min}$ (Fig. 1b). When 400 μ mol/l diazoxide was introduced, insulin release was reduced to basal levels, but remained pulsatile. Also $[\text{Ca}^{2+}]_i$ was decreased to basal but in this case stable levels. In the presence of 11 mmol/l glucose and 400 μ mol/l diazoxide, elevation of the K^+ concentration to 30.9 mmol/l restored the average secretion to the rate observed with 11 mmol/l glucose alone. The amplitude of the pulses tended to be slightly lower than at 11 mmol/l glucose alone, although this difference did not reach statistical significance. There was an initial $[\text{Ca}^{2+}]_i$ peak in response to 30.9 mmol/l K^+ , followed by return to a stably elevated level. Addition of 400 μ mol/l diazoxide alone to perfusion medium containing 3 mmol/l glucose did not affect amplitude or frequency of the insulin pulses (Fig. 2). Basal insulin secretion in the presence of diazoxide was also unaffected by raising

the glucose concentration to 11 mmol/l (Fig. 2) or 20 mmol/l (not shown).

Simultaneous introduction of 400 μ mol/l diazoxide and elevation of the K^+ concentration in medium containing 3 mmol/l glucose caused an almost 30-fold increase of the average insulin release largely due to increased amplitude of the insulin pulses, which maintained their frequency (Table 2). However, the secretory response varied between islets. Both modest responses with or without an initial peak (Fig. 3a and b) and prompt and accentuated increases (Fig. 3c) in pulsatile insulin release were observed. Subsequent elevation of the glucose concentration to 11 mmol/l almost doubled insulin release without affecting the pulse frequency (Table 2). At 20 mmol/l glucose there was a tendency to further stimulation of insulin secretion and decreased frequency of the pulses, but these effects did not reach statistical significance.

In order to assess the influence of prolonged opening of the K_{ATP} channels, glucose-induced insulin release was also studied in islets exposed to 400 μ mol/l diazoxide and 30.9 mmol/l K^+ for 60 min prior to measuring secretion. In the presence of 3 mmol/l glucose, such prolonged exposure tended to enhance secretion less than acute stimulation with 400 μ mol/l diazoxide and elevated K^+ , although this effect could not be established statistically (Tables 2 and 3, Figs 3 and 4). Also these islets displayed variation in their secretory responses (Fig. 4a and b). Elevation of the glucose concentration to 11 mmol/l increased pulsatile insulin release, but the frequency of the pulses was not altered. When the glucose concentration was subsequently augmented to 20 mmol/l, average insulin secretion increased further. This increase appeared to be accomplished by increasing the non-pulsatile component as well as the amplitude of the insulin

Table 2 Insulin release from isolated islets after simultaneous introduction of diazoxide and elevated K^+ . Individual pancreatic islets were perfused in the presence of 3, 11 and 20 mmol/l glucose. Introduction of diazoxide and elevation of K^+ were performed simultaneously. Values are means $\pm$ S.E.M. for eight experiments.

Glucose (mmol/l)	Diazoxide (μ mol/l)	K^+ (mmol/l)	Insulin release	
			pmol·g $^{-1}$ ·s $^{-1}$	Oscillations (per min)
3	—	5.9*	8 $\pm$ 2	0.27 $\pm$ 0.04
3	400	30.9	233 $\pm$ 70†	0.25 $\pm$ 0.05
11	400	30.9	442 $\pm$ 129*	0.21 $\pm$ 0.05
20	400	30.9	674 $\pm$ 206	0.16 $\pm$ 0.04

* $P < 0.05$, † $P < 0.01$ versus preceding perfusion condition.

Figure 3 Glucose-induced insulin release from individual islets after simultaneous introduction of diazoxide and elevated K^+ . Individual mouse islets were perfused in the presence of 3, 11 and 20 mmol/l glucose. The arrow indicates when 400 μ mol/l diazoxide were added to the perfusion medium and K^+ elevated to 30.9 mmol/l. Secretory pattern obtained in $n = 3$ (a), $n = 2$ (b) and $n = 3$ (c) islets.

Table 3 Insulin release from isolated islets in the presence of diazoxide and elevated K^+ . Individual pancreatic islets were perfused in the presence of 3, 11 and 20 mmol/l glucose, 400 μ mol/l diazoxide and 30.9 mmol/l K^+ . Islets were perfused in the presence of 3 mmol/l glucose, 400 μ mol/l diazoxide and elevated K^+ for 60 min before analysis of insulin of the perfusate began. Values are means $\pm$ S.E.M. for five experiments.

Glucose (mmol/l)	Diazoxide (μ mol/l)	K^+ (mmol/l)	Insulin release	
			pmol $\cdot$ g $^{-1}\cdot$ s $^{-1}$	Oscillations (per min)
3	400	30.9	152 $\pm$ 70	0.21 $\pm$ 0.05
11	400	30.9	219 $\pm$ 76*	0.22 $\pm$ 0.04
20	400	30.9	385 $\pm$ 96†	0.12 $\pm$ 0.05§

* $P < 0.05$, † $P < 0.01$ versus preceding perfusion condition; § $P < 0.01$ versus perfusion at 3 mmol/l glucose.

pulses. Furthermore, the rise in insulin release at 20 mmol/l was associated with a decrease in pulse frequency as compared with that at 3 mmol/l glucose.

Discussion

The present measurements of insulin release and $[Ca^{2+}]_i$ were not performed in parallel, which precludes a direct correlation of the data. The $[Ca^{2+}]_i$ oscillations were seemingly more complex than the insulin pulses, probably as a result of the much more rapid sampling in the $[Ca^{2+}]_i$ measurements. Apart from slow oscillations of $[Ca^{2+}]_i$ with frequencies similar to those of the presently studied insulin pulses, this sampling captures the fast type of $[Ca^{2+}]_i$ oscillations

Figure 4 Glucose-induced insulin release from individual islets in the presence of diazoxide and elevated K^+ . Individual mouse islets were perfused in the presence of 3, 11 and 20 mmol/l glucose, 400 μ mol/l diazoxide and 30.9 mmol/l K^+ . Islets were perfused in 3 mmol/l glucose, 400 μ mol/l diazoxide and 30.9 mmol/l K^+ for 60 min prior to analyzing insulin secretion. Secretory patterns obtained in $n = 2$ (a) and $n = 3$ (b) islets.

corresponding to the electrophysiological burst pattern (30). With higher time resolution of the insulin sampling a fast pattern of pulsatile secretion can also be resolved from single islets (14), but since bursting is not synchronized in the pancreas (31) the rapid pattern cannot influence the circulating levels of the hormone. The slow frequency component was dominating in the present $[Ca^{2+}]_i$ measurements although it was slightly higher than that found for pulsatile insulin secretion. However, the aim of the present studies was not to confirm the previously established correlation between pulsatile insulin secretion and $[Ca^{2+}]_i$ (13, 14) but rather to study secretion under conditions when $[Ca^{2+}]_i$ remains stable without oscillations.

Whereas glucose-stimulated insulin secretion via the K_{ATP} channel-dependent pathway is associated with variations in the K_{ATP} channel activity (32) and oscillations of $[Ca^{2+}]_i$ (8, 9, 13–15, 17), no such oscillations are generated by the K_{ATP} channel-independent pathway. Since $[Ca^{2+}]_i$ is the most important determinant of exocytosis of insulin granules (7), it is tempting to associate the two glucose-signaling pathways with pulsatile and non-pulsatile insulin secretion. However, although $[Ca^{2+}]_i$ oscillations are important for the generation of pulsatile insulin release (8, 9, 13–15, 17), there is evidence that pulsatile insulin release occurs also in the absence of such oscillations in $[Ca^{2+}]_i$ (20, 25–27).

We now demonstrate generation of pulsatile insulin secretion via the K_{ATP} channel-independent pathway. Our results are in conflict with those of another study, in which no pulsatile insulin secretion was observed during glucose stimulation of single islets when $[Ca^{2+}]_i$ was kept stable by K^+ depolarization in the presence of diazoxide (24). It is therefore important to note that these authors have never observed pulsatile secretion when the K_{ATP} channel-dependent pathway is operating under physiological conditions. However, pulsatile secretion via the K_{ATP} channel-dependent pathway is observed in the presence of 10 mmol/l Ca^{2+} (15), which is almost 10-fold higher than in a physiological medium. Apart from increasing the secreted amounts of insulin sufficiently to exceed the detection limit of the assay, the elevated Ca^{2+} concentration retards the electrophysiological burst pattern (33, 34), whose frequency approaches that of pulsatile secretion under physiological conditions (35). Since bursting is not synchronized between islets (31) it is questionable if the pulsatile secretion occurring in parallel with the retarded bursts has any relevance for pulsatile insulin secretion from the pancreas. The present study deals primarily with the insulin oscillations with 3–7 min duration. These oscillations, which can readily be detected from the isolated islet, the perfused pancreas and in the blood (1, 3–5, 36), are believed to be important for the hypoglycemic effect of the hormone (37, 38).

The present data indicate that glucose-induced insulin release via the K_{ATP} channel-independent pathway involves both increases of pulse amplitude and the non-pulsatile component of secretion. This observation is not surprising if pulsatile insulin secretion exhibits a dual dependency on metabolism and $[Ca^{2+}]_i$ (27). Exocytosis of insulin granules is triggered by the rise of $[Ca^{2+}]_i$ (7), but is also an energy-dependent process requiring ATP (39). As previously suggested, oscillations in metabolism or $[Ca^{2+}]_i$ may consequently generate pulsatile secretion when the other factor remains stable (27). However, the pulsatile component of secretion is more pronounced when the oscillations of metabolism and $[Ca^{2+}]_i$ act in synergy. The principal that pulsatile secretion can be generated by oscillations in either metabolism or $[Ca^{2+}]_i$ was recently confirmed (40). Other possible explanations for the increase in non-pulsatile secretion may include direct effects by diazoxide on mitochondrial function (41) and prolonged elevation of $[Ca^{2+}]_i$.

The amount of insulin secreted from the individual islets in the presence of diazoxide and depolarizing K^+ concentration varied in this study. This heterogeneous secretory rate may result from different proportions of non- β -cells between the islets (42). Although the *ob/ob* islets contain a high proportion of β -cells, variations in the content of glucagon-producing α -cells could markedly affect insulin secretion since glucagon potentially amplifies glucose-stimulated insulin secretion by raising cAMP (43). Variations in the content of somatostatin-producing δ -cells may also have pronounced effects since this hormone is a potent inhibitor of both glucagon and insulin secretion (44). Metabolic and secretory heterogeneity has been demonstrated also in dispersed β -cells (45), in single islets (46, 47), groups of islets taken from different parts of the pancreas (48–50), and when perfusing the dorsal and ventral part of the pancreas (51).

Like glucose signaling via the K_{ATP} channel-dependent pathway, the present data indicate that also the K_{ATP} channel-independent pathway produces amplitude-regulated pulsatile release of insulin. The finding is important, since both pathways are present in human β -cells (23) and contribute to secretion under physiological conditions.

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