Rapid oscillation of insulin release by the rat pancreatic islets under stringent Ca^{2+}-free conditions

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Abstract

Oscillation of insulin release by the pancreatic islets was evaluated under stringent Ca^{2+}-free conditions for the first time. Isolated single rat islets were exposed to 16·7 mM glucose in the presence of 1·9 mM Ca^{2+}, or under the stringent Ca^{2+}-free conditions (Ca^{2+} omission with 1 mM EGTA, 6 μM forskolin and 100 nM phorbol 12-myristate 13-acetate). Fifteen minutes after the initiation of glucose stimulation, effluent was collected at a 6-s interval, insulin was determined in duplicate by a highly sensitive insulin radioimmunoassay, and oscillation and pulsatility of release statistically analyzed. Significant oscillation of insulin release was observed in all islets irrespective of presence and absence of Ca^{2+}. Significant pulsatility of release was detected in 7 of 11 islets in the presence of Ca^{2+} and three of six islets in the absence of Ca^{2+}. In conclusion, high glucose elicits oscillatory insulin release both in the presence and absence of extracellular Ca^{2+}.

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Introduction

Insulin release by individual isolated pancreatic islets was first investigated by Beigelman *et al.* (Beigelman *et al.* 1973*a*,*b*, 1976). Since then, data on insulin release by the single islets have been accumulated (Beigelman *et al.* 1973*a*,*b*, 1976, Rosario *et al.* 1986, Bergsten & Hellman 1993*a*,*b*, Gilon *et al.* 1993, Bergsten *et al.* 1994, Gilon & Henquin 1995, Bergsten 1995, Zaitsev *et al.* 1995, Westerlund *et al.* 1996, 1997), and it is now well established that pancreatic islets are the smallest units of coordinated insulin secretion. Even in the initial studies, it was noticed that a high concentration of glucose elicits pulsatile insulin release by single islets (Beigelman *et al.* 1973*b*, 1976). Especially in the presence of high concentration of Ca^{2+}, temporal coupling between oscillations of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]i$) and insulin release is so tight (Gilon *et al.* 1993, Gilon & Henquin 1995) that it looks apparent that the former is driving the latter. However, glucose stimulation of insulin release is not solely mediated by elevation of $[Ca^{2+}]i$ (Aizawa *et al.* 1992, 1994, Gembal *et al.* 1992, Sato *et al.* 1992, Komatsu *et al.* 1997, Aizawa *et al.* 1998, Henquin *et al.* 1998). In particular, glucose potently augments insulin release even under stringent Ca^{2+}-free conditions (Komatsu *et al.* 1995, 1996, 1997, Aizawa *et al.* 1998, Sato *et al.* 1998, Yajima *et al.* 1999). In this communication, we report that glucose-stimulated insulin release occurs in a fine oscillatory manner in the order of seconds under the Ca^{2+}-free conditions as well. The data implies that glucose produces non-Ca^{2+} signal or signals which promote coordination of islet β cells leading to oscillation of insulin release.

Materials and Methods

Pancreatic islets were obtained from male, adult Wistar rats by collagenase dispersion and directly hand-picked (Sato *et al.* 1992, Aizawa *et al.* 1994, 1996, Taguchi *et al.* 1995, Asanuma *et al.* 1997) and used for the experiments within 2 h. Exposure of the islets to Ficoll was intentionally avoided as previously recommended (Beigelman *et al.* 1973*a*). In this study, islets with the diameter of 200–300 μm were used. For isolation and subsequent washing of the islets, Krebs–Ringer bicarbonate (KRB) buffer containing (in mM) NaCl 118·4, KCl 4·7, $MgSO_4$ 1·2, KH_2PO_4 1·2, $CaCl_2$ 1·9, $NaHCO_3$ 25 (equilibrated with 5% CO_2–95% O_2, pH 7·4), glucose 5·5, and 0·1% BSA was used, and the experiments were carried out at 37 °C using the KRB buffer containing 0·2% BSA as described (Sato *et al.* 1992, Aizawa *et al.* 1994, 1996, Taguchi *et al.* 1995, Asanuma *et al.* 1997) with minor modifications. The modifications were mostly down-sizing of the perifusion system: the volume of chamber and flow rate were

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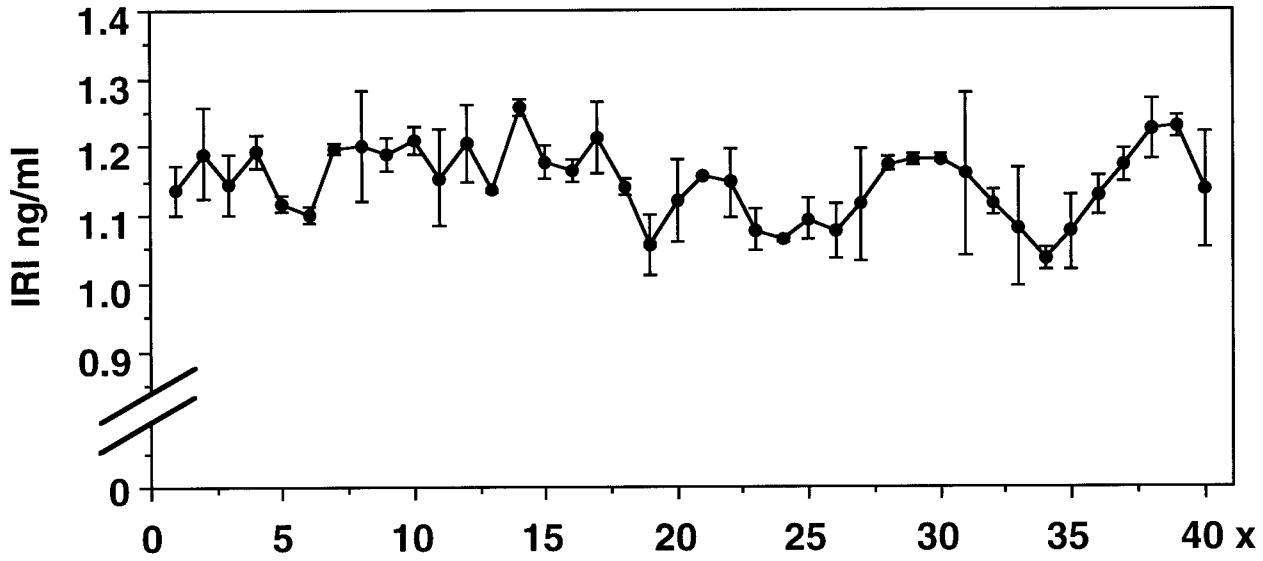

Figure 1 Assay variation in insulin radioimmunoassay. IRI stands for immunoreactive insulin. KRB buffer containing a known concentration of insulin was repetitively assayed in duplicate for 40 times. The evaluation was done on non-perifused sample, and the values were plotted to simulate variation of IRI in the real experiments. The values are means ± ranges of the duplicate assay. See the Materials and Methods section for details. The conversion factor for ng to nmol is 0·0001739.

reduced to 20 μl and 180 μl/min respectively. Two 5 ml syringes were mounted on two identical syringe pumps (Pump 22, Harvard, South Natick, MA, USA), and one was used for pre-wash period and the other used for glucose stimulation period (see below). The accuracy of the flow was ± 1%. Sephadex G-10 was used as supporting matrix and the plastic mesh, not a glass bead, was placed at the bottom of the chamber to prevent outflow of Sephadex. Pressure of the perifusion circuit was monitored in each experiment, and it was <15 cm H_2O in all. Before starting glucose stimulation, the islets were perifused with KRB buffer containing 3 mM glucose for 30 min. In some experiments, Ca^{2+}-omitted KRB buffer with 1 mM EGTA (Ca^{2+}-free KRB buffer) was used as described (Komatsu *et al.* 1995). In brief, the islets were first incubated in the Ca^{2+}-free KRB buffer containing 3 mM glucose for 60 min at 37 °C. In this case, before exposure to high glucose, the islets were perifused with the Ca^{2+}-free KRB buffer containing 3 mM glucose, 6 μM forskolin and 100 nM phorbol 12-myristate 13-acetate (TPA) for 30 min. Then, the Ca^{2+}-free buffer was switched to the one with 16·7 mM glucose with the continuous presence of forskolin and TPA.

Perifusate was collected as 7 μl drops. Before the determination of oscillation, effluent was collected at 1–2 min intervals (see below). From 15 min after the initiation of glucose stimulation, effluent was collected at a 6-s interval, i.e. mostly two drops per tube, and the oscillation of insulin release was determined. To minimize binding of insulin to the tube wall, silicon-coated glass tubes were used for collection of the effluent, the tubes were centrifuged at 4 °C immediately after the collection and placed on ice, and insulin assay was performed on the same day. Insulin RIA was performed in duplicate (with a sampling volume of 6·5 μl each) using high sensitivity rat insulin RIA kits (Linco Research Inc., St Louis, MO, USA): the minimum detectable amount of insulin was 1·0 pg/tube, the half-maximum displacement occurred at approximately 10 pg/tube, and the intra-assay coefficient of variation ((standard deviation/mean) × 100, CV) was 6·1% in single assay and 4·6% in duplicate assay, each performed by ourselves. To avoid inter-assay variation, all samples from one experiment were assayed together in one assay. Forskolin and TPA were obtained from Sigma (St Louis, MO, USA).

Statistical analysis was performed using Wald–Wolfowitz runs test, Mann–Whitney U-test, and χ^2 test (StatView, SAS Institute Inc., Cary, NC, USA), and the partial autocorrelation plot (SYSTAT, SPSS, Chicago, IL, USA). $P<0·05$ was considered significant. Data are presented as means ± S.E.S unless otherwise indicated.

Results

Evaluation of non-specific variation in the insulin RIA

To critically evaluate the degree of assay variation in the RIA, KRB buffer with 0·2% BSA containing a known concentration of insulin standard was consecutively assayed in duplicate with a 6·5 μl sampling volume for 40 times. The values are plotted (Fig. 1) to simulate the experimental oscillation shown in other figures (Figs 3A and 4A

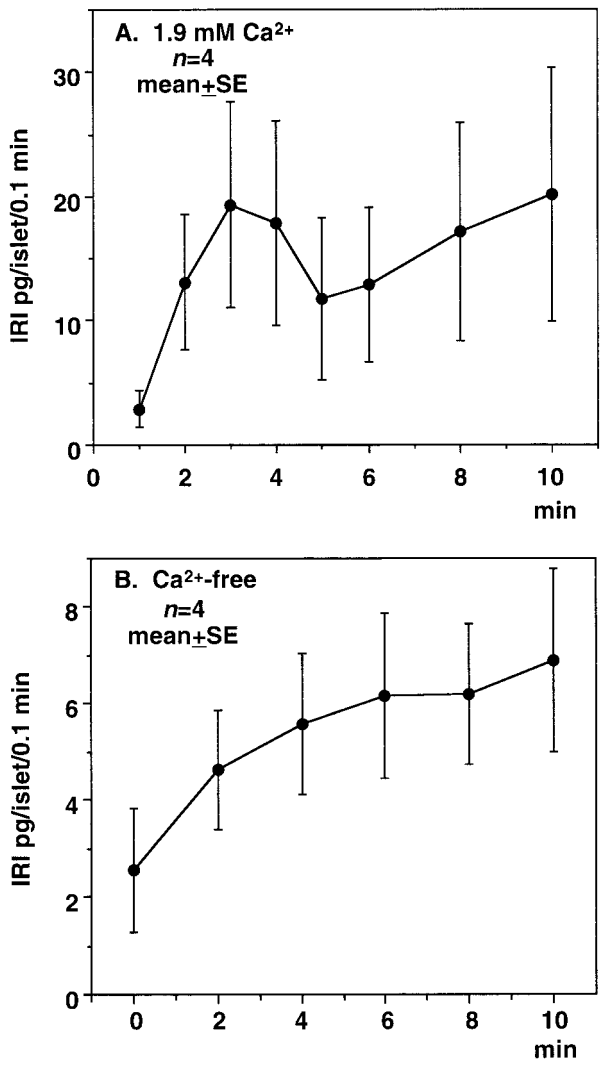

Figure 2 Insulin released by the single islets during the initial period of exposure to 16·7 mM glucose in the presence of 1·9 mM Ca^{2+} (A) or under the stringent Ca^{2+}-free conditions (Ca^{2+} omission with 1 mM EGTA, 6 μM forskolin and 100 nM TPA) (B). The data shown in this figure were obtained by the single assay with a sampling volume of 50 μl. The conversion factor for pg/islet to nmol/islet is 0·1739. IRI, immunoreactive insulin.

below). The variation obtained in this experiment was compared with that obtained in the real perifusion experiments by Wald–Wolfowitz runs test to know the significance of oscillation in each experiment (see below).

Insulin release in the presence of regular Ca^{2+}

Insulin release was clearly increased in 10 out of 11 islets upon exposure to 16·7 mM glucose (Fig. 2A). From 15 min after the initiation of glucose stimulation, perifusate was collected at a 6-s interval for 3–5 min (Fig. 3A). Statistically significant oscillation of insulin release, compared with the non-specific assay variation (Fig. 1), was detected in all islets responded to glucose. The CV value determined as an index of the degree of oscillation was 19·3 ± 1·4% (n=10). In seven of them, significant pulsatility was detected by the partial autocorrelation plot (Fig. 3B). There were positive deflections with the intervals being 6 s (in five islets), and 18 and 30 s (in one islet each), and negative deflections with the intervals being 48 s (one islet), 36 and 54 s (one islet), and 54 s (one islet). In some islets, pulsatility with two different frequencies was detected as in Fig. 3B, II and IV.

Insulin release under the stringent Ca^{2+}-free conditions

All six islets responded to 16·7 mM glucose with gradual increase in the rate of insulin release under stringent Ca^{2+}-free conditions (Ca^{2+} omission with 1 mM EGTA, 6 μM forskolin and 100 nM TPA) as previously reported (Komatsu *et al.* 1995, 1996, Sato *et al.* 1998, Yajima *et al.* 1999) (Fig. 2B). Fifteen minutes after the initiation of glucose stimulation, perifusate was collected at a 6-s interval for 3–5 min and oscillation of insulin release was determined (Fig. 4A). Statistically significant oscillation of insulin release, compared with the non-specific assay variation (Fig. 1), was detected in all six islets. The CV value obtained as an index of degree of oscillation was 13·3 ± 3·4% (n=6). Using the partial autocorrelation plot, a significant pulse was detected in three islets (Fig. 4B, I–III). There was a 6-s interval positive deflection (Fig. 4B, I), a 30-s interval negative deflection (Fig. 4B, II), and 18- and 84-s interval negative deflections (Fig. 4B, III).

The CV value in the experiments under the stringent Ca^{2+}-free conditions was smaller than that in the experiments with 1·9 mM Ca^{2+} (13·3 ± 3·4 vs 19·3 ± 1·4%). However, the difference did not reach the statistically significant level (P=0·059).

Discussion

The insulinotropic action of glucose can be divided into at least three categories including (1) K^+ATP channel-*dependent*; (2) K^+ATP channel-*independent* and Ca^{2+}-*dependent*; and (3) K^+ATP channel-*independent* and Ca^{2+}-*independent* (Komatsu *et al.* 1997, Aizawa *et al.* 1998). The physiological role of K^+ATP channel-independent glucose actions cannot be dismissed for *in vivo* regulation of insulin secretion where not only glucose but also incretins, amino acids, fatty acids, and neurotransmitters all act together to elicit insulin secretion (Aizawa *et al.* 1998, Yajima *et al.* 1999, Komatsu *et al.* 1999). In the present study, we tried to detect rapid oscillation and pulsatility of insulin release upon glucose stimulation, both in the presence of regular concentration of extracellular Ca^{2+} and under stringent Ca^{2+}-free conditions. In the former condition, glucose is

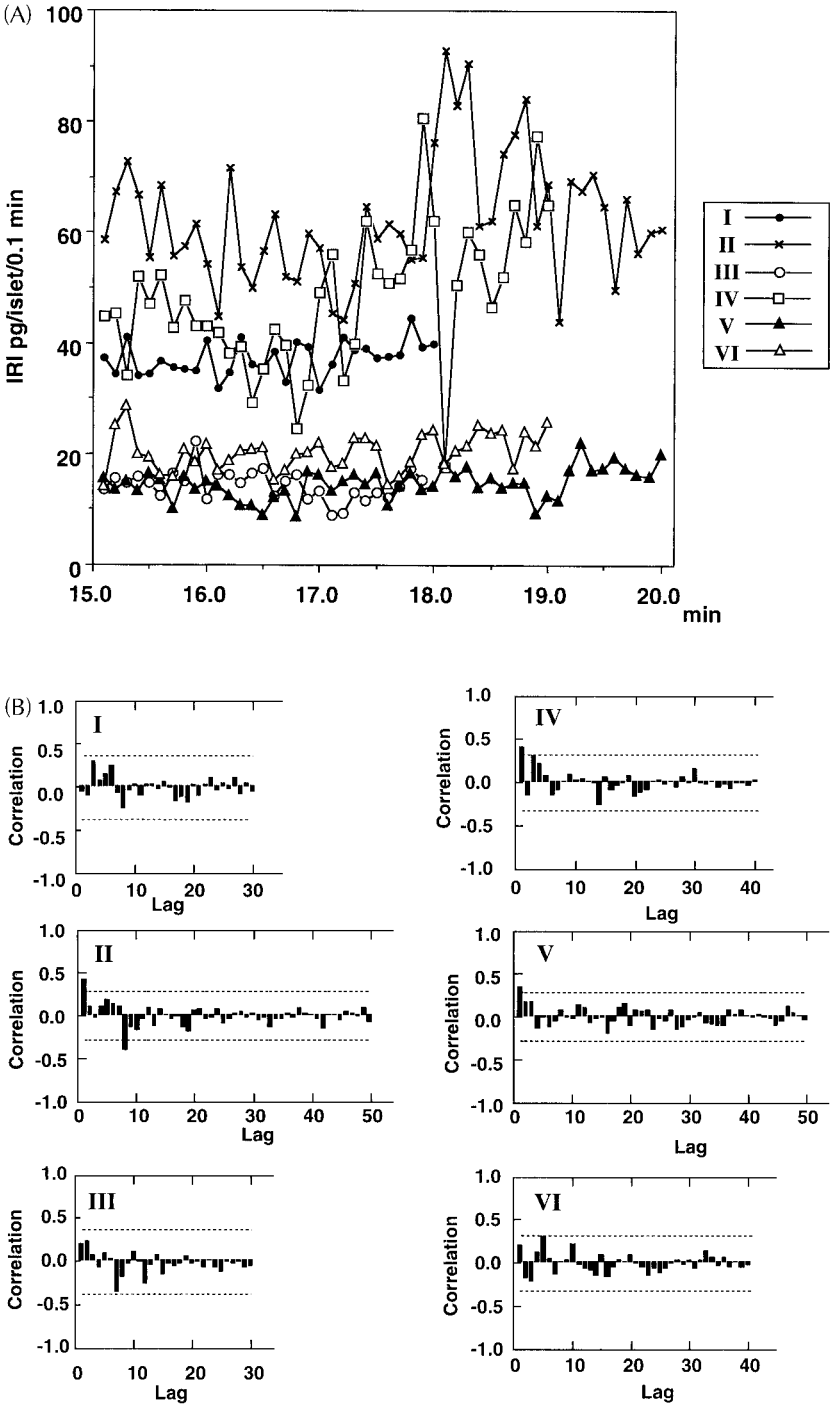

Figure 3 Oscillation of insulin release in the presence of 1·9 mM Ca^{2+}. Representative results from six islets are shown. Actual fluctuation of insulin concentration in the effluent is shown in (A). IRI stands for immunoreactive insulin, and the conversion factor for pg/islet to nmol/islet is 0·1739. The glucose concentration of the perifusate was raised from 3 to 16·7 mM at time 0. Fifteen minutes after the initiation of glucose stimulation, perifusate was collected at a 6-s interval for the duration of 5 min (II and V), 4 min (IV and VI), or 3 min (I and III). Analyses of pulsatility by the partial autocorrelation plot are presented in (B). Each Roman numeral in (A) and (B) corresponds, i.e. the partial autocorrelation plot in (B) I was obtained from the raw data in (A) I, (B) II from (A) II, and so on. The horizontal dotted lines in (B) indicate the level of $P=0·05$. Hence, if the height of a bar exceeds the dotted line, a statistically significant pulse exists at that lag (time point). Significant pulses were detected in (B), II, IV, V, and VI, but not in I and III. Upward and downward bars indicate positive and negative deflections respectively. One lag in (B) corresponds to 6 s.

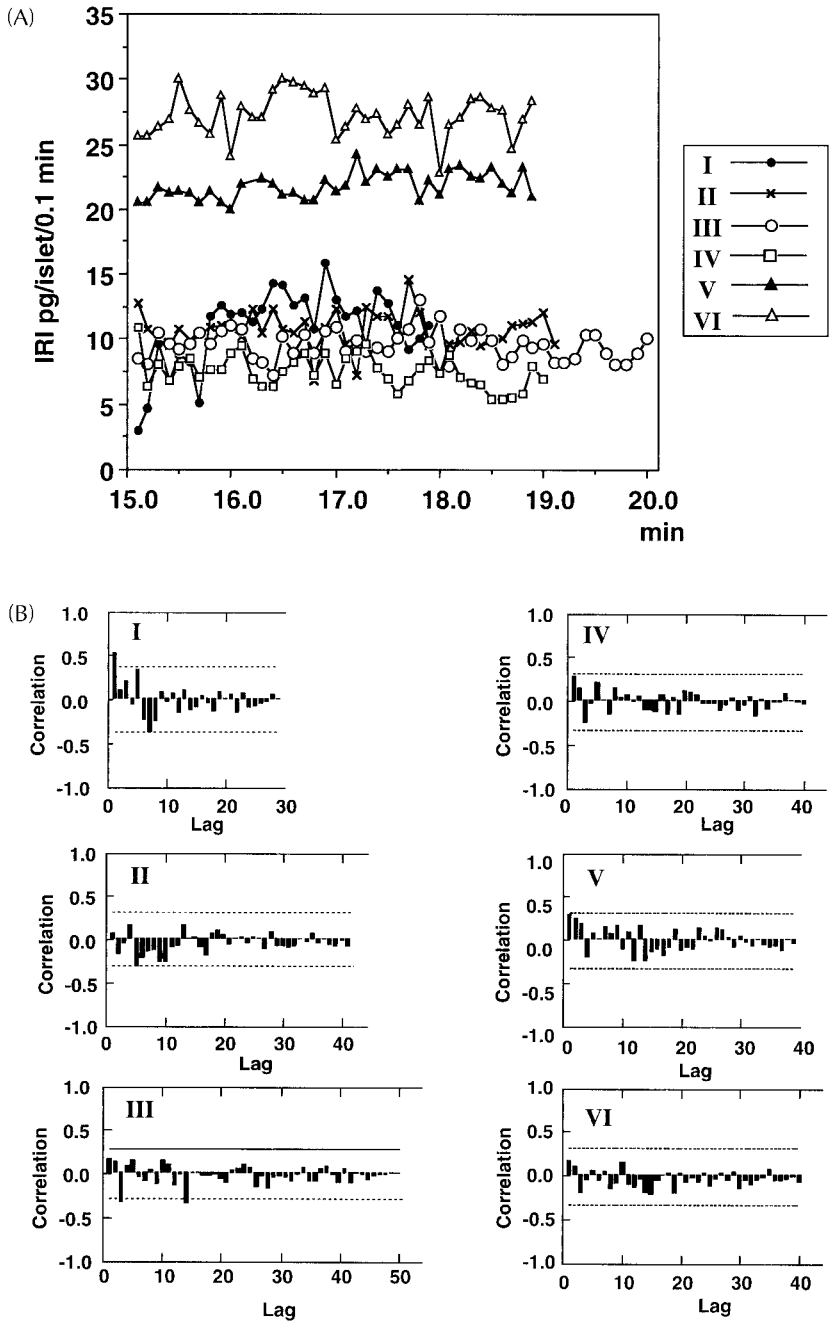

Figure 4 Oscillation of insulin release under the stringent Ca^{2+}-free conditions. The actual fluctuation of insulin concentration in the effluent is shown in (A). IRI stands for immunoreactive insulin, and the conversion factor for pg/islet to nmol/islet is 0·1739. Glucose concentration of perifusate was raised from 3 to 16·7 mM under the stringent Ca^{2+}-free conditions (Ca^{2+} omission with 1 mM EGTA, 6 μM forskolin and 100 nM TPA) at time 0. Fifteen minutes after the initiation of glucose stimulation, the perifusate was collected at a 6-s interval for the duration of 5 min (III), 4 min (II, IV, V and VI), or 3 min (I). Analyses of pulsatility by the partial autocorrelation plot are presented in (B). Each Roman numeral in (A) and (B) corresponds, i.e. the partial autocorrelation plot in (B) I was obtained from the raw data in (A) I, (B) II from (A) II, and so on. Horizontal dotted lines in (B) indicate the level of $P=0·05$. Hence, if the height of a bar exceeds the dotted line, a statistically significant pulse exists at that lag (time point). Significant pulses were detected in (B), I, II, and III, but not in IV, V, and VI. Upward and downward bars indicate positive and negative deflections respectively. One lag in (B) corresponds to 6 s.

stimulating insulin release through 'K^+ATP channel-dependent' and 'K^+ATP channel-independent and Ca^{2+}-dependent' pathways, whereas, in the latter condition, the hexose is stimulating insulin release selectively through 'K^+ATP channel-independent and Ca^{2+}-independent' pathway. We used freshly isolated rat islets as material and insulin concentration was determined by using a highly sensitive rat insulin RIA. The most prominent finding in our study is that a high concentration of glucose elicits rapid, oscillatory insulin release even in the stringent Ca^{2+}-free conditions.

Rapid, oscillatory insulin release was found also with 11·1 mM glucose in regular KRB buffer (data not shown). Here, islets kept for 4 h after isolation in regular KRB buffer were employed. The finding strongly suggests that rapid oscillation of insulin release stated above occurs at a physiological concentration of glucose, and that it is not a unique phenomenon to the islets used rapidly after isolation.

Furthermore, we revealed the following intriguing facts. In freshly isolated islets (present data), the amplitude of oscillation was lower and frequency of pulsatility was higher than in cultured mouse islets (Gilon *et al.* 1993, Gilon & Henquin 1995, Bergsten *et al.* 1994, Bergsten 1995, Zaitsev *et al.* 1995, Westerlund *et al.* 1996, 1997), indicating that the proportion of functionally coupled β cells in the islet is lower in fresh islets. In other words, in cultured islets, a larger fraction of β cells/islet appears to be functionally coupled and secreting insulin synchronously, which may be due to a loss of centrally located cells after culture. In the present study, the degree of oscillation was smaller in the absence of Ca^{2+}, although the difference did not reach the statistically significant level. Also, a positive deflection of release with a short duration was predominantly found in the presence of Ca^{2+}. The differences may be mostly, if not entirely, due to elimination of Ca^{2+} signals under the stringent Ca^{2+}-free conditions.

Pulsatile insulin release by the single pancreatic islets has previously been evaluated mostly using the islets from the *ob/ob* mouse (Rosario *et al.* 1986, Bergsten & Hellman 1993*a*,*b*, Bergsten *et al.* 1994, Bergsten 1995, Zaitsev *et al.* 1995, Westerlund *et al.* 1996, 1997) whose islets are hypertrophic and the β cell is under intense stimulation because the animal is markedly insulin resistant due to obesity caused by leptin deficiency (Zhang *et al.* 1994). The islets from normal mice were used in the initial studies (Beigelman *et al.* 1973*a*,*b*, 1976) but more recently only in two studies in which the islets were exposed to 10 mM glucose overnight in culture media and subsequently tested in the presence of a very high concentration of Ca^{2+}, that is 10 mM (Gilon *et al.* 1993, Gilon & Henquin 1995). Regarding extracellular Ca^{2+} concentration ($[Ca^{2+}]o$), the previous experiments were carried out in the presence of 10 mM (Rosario *et al.* 1986, Gilon *et al.* 1993, Gilon & Henquin 1995), approximately 2·6 mM (Rosario *et al.* 1986, Bergsten *et al.* 1994, Bergsten 1995), or 1·3 mM (Bergsten & Hellman 1993*a*,*b*, Zaitsev *et al.* 1995, Westerlund *et al.* 1996, 1997) Ca^{2+}. The majority of studies were carried out using cultured islets (Gilon *et al.* 1993, Bergsten *et al.* 1994, Bergsten 1995, Gilon & Henquin 1995, Zaitsev *et al.* 1995, Westerlund *et al.* 1996, 1997) where the central necrosis is unavoidable, especially in larger islets. Nevertheless, from the previous studies, it can be summarized that synchronization of changes in $[Ca^{2+}]i$ and insulin release was evident in the presence of higher $[Ca^{2+}]o$ concentrations (Gilon *et al.* 1993, Bergsten *et al.* 1994, Bergsten 1995, Gilon & Henquin 1995) and dissociation of the two was found in the presence of lower $[Ca^{2+}]o$ (Zaitsev *et al.* 1995, Westerlund *et al.* 1996, 1997), suggesting high $[Ca^{2+}]o$ is a prerequisite for the tight temporal coupling of $[Ca^{2+}]i$ and insulin release to occur. In extrapolation of the data obtained in the presence of lower $[Ca^{2+}]o$ (Zaitsev *et al.* 1995, Westerlund *et al.* 1996, 1997), our finding that oscillation of insulin release occurs even under stringent Ca^{2+}-free conditions is not totally unexpected.

There are more than several thousand β cells in each islet (Pipeleers *et al.* 1992) so that non-oscillatory insulin release is expected if all β cells in the islet secrete insulin, say, randomly or with randomly distributed rhythmicity. Glucose-induced oscillatory elevation of $[Ca^{2+}]i$ which propagates from one β cell to another may be causing an oscillatory insulin release by the islets as proposed, especially in the presence of high $[Ca^{2+}]o$ (Gilon *et al.* 1993, Bergsten *et al.* 1994, Bergsten 1995, Gilon & Henquin 1995). However, oscillatory elevation of $[Ca^{2+}]i$ cannot be a driving force for the oscillation of insulin release under the stringent Ca^{2+}-free conditions because glucose does not cause elevation of $[Ca^{2+}]i$ (Komatsu *et al.* 1995, 1996). Therefore, under stringent Ca^{2+}-free conditions, functional coupling among β cells may be produced by propagation of non-Ca^{2+} ionic events (Atwater *et al.* 1996), glucose metabolites (Henquin *et al.* 1998, Tornheim 1997) and/or yet unidentified messengers, which may eventually control the rate of insulin release at the level of exocytosis *per se* (priming, docking and/or fusion) (Hisatomi *et al.* 1996, Rorsman 1997, Daniel *et al.* 1999).

We established oscillatory, coordinated insulin release under stringent Ca^{2+}-free conditions. The data supports the idea that high concentration of glucose generates Ca^{2+} and non-Ca^{2+} pulsatile signals at various steps of stimulus–secretion coupling, and the hierarchy of them decides the eventual pattern of insulin exocytosis (Henquin *et al.* 1998). Further studies are needed to elucidate the entire picture of mediation of oscillatory insulin release.

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