FEBS 26657

FEBS Letters 530 (2002) 215–219

Time and amplitude regulation of pulsatile insulin secretion by triggering and amplifying pathways in mouse islets

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Received 22 August 2002; accepted 17 September 2002

First published online 1 October 2002

Edited by Jacques Hanoune

Abstract **Glucose-induced insulin secretion is pulsatile. We investigated how the triggering pathway (rise in β-cell $[Ca^{2+}]_i$) and amplifying pathway (greater Ca^{2+} efficacy on exocytosis) influence this pulsatility. Repetitive $[Ca^{2+}]_i$ pulses were imposed by high K^+ + diazoxide in single mouse islets. Insulin secretion (measured simultaneously) tightly followed $[Ca^{2+}]_i$ changes. Lengthening $[Ca^{2+}]_i$ pulses increased the duration but not the amplitude of insulin pulses. Increasing glucose (5–20 mmol/l) augmented the amplitude of insulin pulses without changing that of $[Ca^{2+}]_i$ pulses. Larger $[Ca^{2+}]_i$ pulses augmented the amplitude of insulin pulses at high, but not low glucose. In conclusion, the amplification pathway ensures amplitude modulation of insulin pulses whose time modulation is achieved by the triggering pathway.**

Key words: Insulin secretion; Oscillation; Glucose; Calcium; Pancreatic islet; Diabetes

1. Introduction

Glucose precisely regulates insulin secretion by producing triggering and amplifying signals in β-cells [1,2]. A rise in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$), resulting from membrane depolarization and Ca^{2+} influx is the signal that triggers exocytosis of insulin granules [3–8]. The nature of the amplifying signal is still disputed [7–11] but there is strong evidence that this pathway serves to augment the efficiency of Ca^{2+} on exocytosis [1].

Insulin secretion is pulsatile [12–14]. In normal mouse and human islets, $[Ca^{2+}]_i$ regularly oscillates during glucose stimulation [15–22]. These oscillations of $[Ca^{2+}]_i$, driven by waves of membrane potential changes, are accompanied by synchronous pulses of insulin secretion [16–18]. Moreover, imposed $[Ca^{2+}]_i$ pulses, induced by repetitive depolarizations with high extracellular K^+, also trigger synchronous pulses of insulin secretion, whereas sustained elevation of $[Ca^{2+}]_i$ causes sustained insulin secretion [23–25]. These observations suggest that $[Ca^{2+}]_i$ is the instantaneous regulator of the rate of insulin secretion. However, insulin secretion also oscillates in rat islets [26,27], although β-cells are continuously depolarized and $[Ca^{2+}]_i$ steadily elevated by glucose [22]. Dissociations between $[Ca^{2+}]_i$ and insulin secretion oscillations have also been reported in leptin-deficient *ob/ob* mouse islets [28]. It was thus proposed that, in some species or under certain conditions, amplifying signals cyclically produced by glucose can override the continuous triggering action of Ca^{2+} [29].

The amplifying pathway of glucose-induced insulin secretion has been discovered and subsequently characterized by using experimental paradigms holding β-cell $[Ca^{2+}]_i$ stably elevated [1]. It is not known whether the amplifying mechanism remains effective when $[Ca^{2+}]_i$ fluctuates in β-cells and could explain the paradox that insulin secretion pulses mainly increase in amplitude when the glucose concentration is raised whereas the concomitant $[Ca^{2+}]_i$ oscillations increase in duration, not in amplitude [15,18,22,30]. This was the question addressed in the present study by using single mouse islets in which $[Ca^{2+}]_i$ and metabolism were experimentally controlled. Thus, diazoxide was used to avoid that changes in glucose influence $[Ca^{2+}]_i$ by affecting the membrane potential, and size-controlled, regular $[Ca^{2+}]_i$ pulses were imposed by repetitive increases and decreases of the extracellular K^+ concentration. Insulin secretion was measured simultaneously from the same islets [23,24].m:/Typ/

2. Materials and methods

2.1. Preparation and solutions

Islets were aseptically isolated by collagenase digestion of the pancreas of female NMRI mice, selected by hand-picking and cultured for 1 day in RPMI 1640 medium containing 10 mmol/l glucose [23]. The control medium used for islet isolation contained (mmol/l) 120 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 24 $NaHCO_3$, 10 glucose, and 1 mg/ml bovine serum albumin. It was gassed with O_2/CO_2 (94/6) to maintain a pH of 7.4. The same medium with 0.1 mmol/l diazoxide was used for all experiments. When the concentration of KCl was increased to 30 mmol/l, that of NaCl was decreased, and when the concentration of $CaCl_2$ was decreased, that of $MgCl_2$ was increased accordingly to maintain iso-osmolarity.

2.2. Combined measurements of $[Ca^{2+}]_i$ and insulin secretion

Cultured islets were loaded with fura-PE3 during 2 h of incubation at 37°C in control medium containing 2 μmol/l fura-PE3 acetoxymethyl ester and 5 mmol/l glucose. After loading, one single islet was transferred into a perifusion chamber (110 μl) with a bottom made of a glass coverslip and mounted on the stage of an inverted microscope equipped for $[Ca^{2+}]_i$ measurement [16]. The islet was held in place with a micropipette and perifused at a flow rate of 1.8 ml/min. To induce repetitive $[Ca^{2+}]_i$ pulses in β-cells, the concentration of K^+ was alternated between 4.8 and 30 mmol/l. The medium (37°C) was collected, in fractions of 30 s, just downstream of the islet. Insulin was measured, in duplicate 400 μl aliquots of the effluent fractions, using rat insulin as a standard [23]. At the end of the experiments the islet was recovered and its content in insulin was measured (88.0 ± 4.7 ng, $n = 45$). Insulin secretion could thus be expressed as a percentage of insulin content.

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0014-5793/02/$22.00
PII: S0014-5793(02)03491-9

2.3. Presentation of results

The results are presented as mean traces (±S.E.M.) for four to six experiments with islets obtained from different preparations. The effects of the different types of stimulations were quantified by computing the difference (Δ) between average $[Ca^{2+}]_i$ or insulin secretion rate and baseline during the periods of interest (see legends to figures for details). The statistical significance of differences between means was assessed by Student's *t*-test (paired or unpaired as appropriate) or by analysis of variance followed by a Newman–Keuls test when more than two groups were compared. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Amplification of insulin secretion by glucose during intermittent $[Ca^{2+}]_i$ elevation

Controlled $[Ca^{2+}]_i$ pulses in β-cells were imposed by repetitive depolarizations induced by alternating between 4.8 and 30 mmol/l K^+ every 2 min (Fig. 1A,B). The concentration of extracellular $CaCl_2$ was kept constant at 1 or 2.5 mmol/l throughout, but that of glucose was increased from 5 to 20 mmol/l in the middle of the experiment. This increase produced a consistent, albeit small attenuation of the $[Ca^{2+}]_i$ pulse that was concomitantly imposed. On the average traces, this can be seen as a shoulder in the ascending phase of the $[Ca^{2+}]_i$ pulse occurring between 18 and 20 min (Fig. 1A,B).

Each $[Ca^{2+}]_i$ pulse triggered a pulse of insulin secretion. The increase in glucose concentration augmented the secretory responses. The effect was most marked on the first pulse in 1 mmol/l $CaCl_2$ (Fig. 1A), but was more sustained in 2.5 mmol/l $CaCl_2$ (Fig. 1B). The quantification of the changes in $[Ca^{2+}]_i$ and insulin is shown in the lower panels of Fig. 1. Mean $[Ca^{2+}]_i$ was marginally higher during the last than the first four pulses, but this small difference achieved statistical significance only in islets perifused in low $CaCl_2$ (Fig. 1C). A similar small increase in Ca^{2+} occurs when K^+ pulses are applied at constant glucose throughout or when K^+ is steadily elevated, and appears to reflect a slow drift of apparent $[Ca^{2+}]_i$ with time [23–25]. Contrasting with the quasi-stability of $[Ca^{2+}]_i$ pulses, the amplitude of the insulin pulses was increased two- to three-fold by 20 mmol/l glucose, the relative change being larger during perifusion with 2.5 than 1 mmol/l $CaCl_2$.

3.2. Interactions between $[Ca^{2+}]_i$ and glucose concentration in the amplification of pulsatile insulin secretion

In these experiments, the concentration of glucose was kept

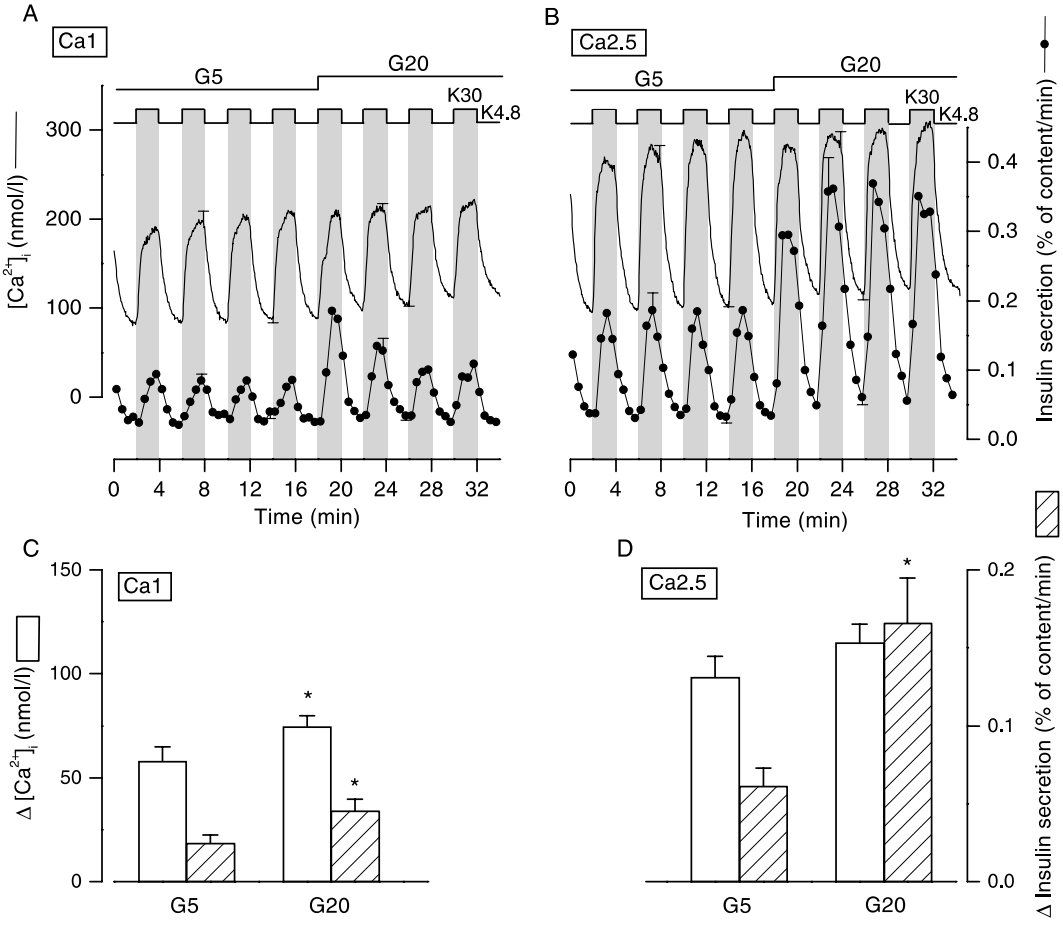

Fig. 1. Amplitude modulation of insulin secretion triggered by $[Ca^{2+}]_i$ pulses in β-cells. A,B: $[Ca^{2+}]_i$ and insulin secretion were measured in the same single islets. The K^+ concentration was alternated between 4.8 and 30 mmol/l every 2 min as indicated, whereas that of glucose (G) was raised from 5 to 20 mmol/l at 18 min. Two depolarizations with K^+ were applied before $[Ca^{2+}]_i$ and insulin measurements were started, hence the decreasing values between 0 and 2 min. C,D: Quantification of the changes (Δ) in $[Ca^{2+}]_i$ and insulin secretion. Δ $[Ca^{2+}]_i$ is the difference between mean $[Ca^{2+}]_i$ from 2 to 18 min or from 18 to 34 min and baseline $[Ca^{2+}]_i$ determined as mean nadir between oscillations in G5. Δ insulin was calculated in a similar way. *$P < 0.05$ or less versus G5. Values are means ± S.E.M. for six islets.

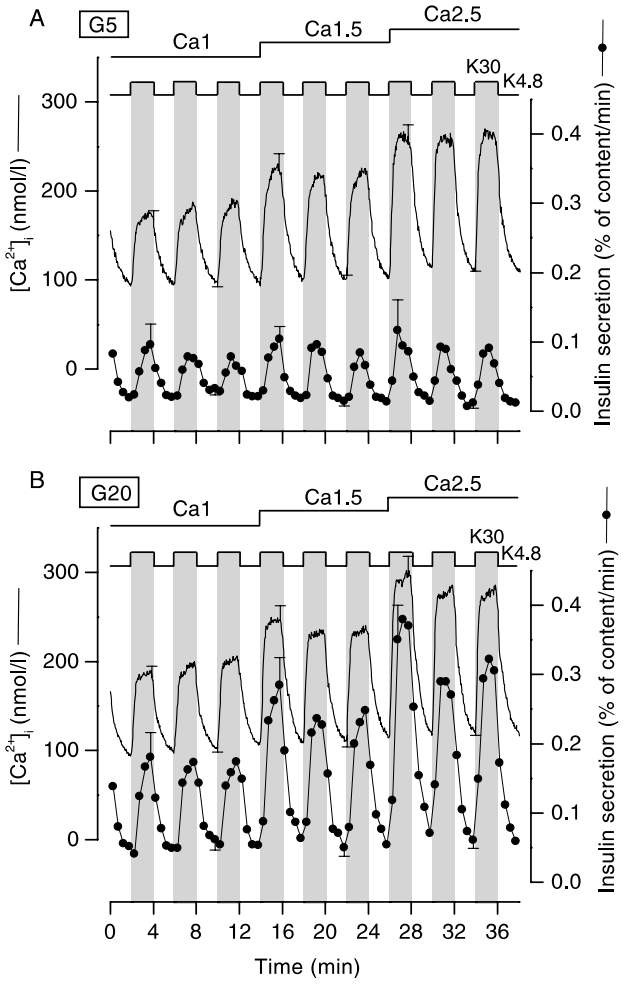

Fig. 2. Influence of glucose on insulin secretion triggered by $[Ca^{2+}]_i$ pulses of increasing amplitude. $[Ca^{2+}]_i$ and insulin secretion were measured in the same single islets. The K^+ concentration was alternated between 4.8 and 30 mmol/l every 2 min as indicated. The glucose concentration was 5 or 20 mmol/l throughout (G5 or G20), and that of extracellular $CaCl_2$ was increased stepwise as indicated. Values are means ± S.E.M. for four islets.

constant at 5 or 20 mmol/l, but the amplitude of the forced $[Ca^{2+}]_i$ pulses was increased by raising extracellular $CaCl_2$ from 1 to 1.5 and 2.5 mmol/l during repetitive depolarizations with 30 mmol/l K^+ (Fig. 2). The impact on insulin secretion was dependent on the ambient glucose concentration. The amplitude of insulin pulses increased with that of $[Ca^{2+}]_i$ pulses in 20 mmol/l glucose (Fig. 2B) but did not significantly change in 5 mmol/l glucose (Fig. 2A). Similar results were obtained in two experiments performed in reverse order, i.e. by decreasing extracellular $CaCl_2$ from 2.5 to 1.5 and 1 mmol/l during the pulses with high K^+ (not illustrated). The quantification of the combined sets of results is presented in Fig. 3A,B, and the relationships between $[Ca^{2+}]_i$ and insulin secretion are shown in Fig. 3C. For any increase in $[Ca^{2+}]_i$, insulin secretion was more strongly stimulated in 20 than 5 mmol/l glucose. Whereas the secretory response increased with $[Ca^{2+}]_i$ in high glucose, no similar dependence was found in low glucose. The difference between the secretory responses at low and high glucose increased with the amplitude of the $[Ca^{2+}]_i$ pulse (Fig. 3C).

3.3. Relative impact of $[Ca^{2+}]_i$ and glucose concentration changes on the pulses of insulin secretion

The preceding experiments have shown that glucose augments the insulin response triggered by imposed $[Ca^{2+}]_i$ pulses with little or no effect on the amplitude of the latter. Under physiological conditions, when the membrane potential of β-cells is not experimentally controlled as here, an increase in glucose from an already stimulatory to an even higher concentration increases the duration of $[Ca^{2+}]_i$ oscillations and barely affects their amplitude [15,18,22,30]. These changes were thus mimicked by changing the duration of the imposed periods of depolarization (Fig. 4). When the glucose concentration was kept at 5 mmol/l, doubling the duration of the forced $[Ca^{2+}]_i$ elevations lengthened the periods of insulin secretion but did not influence their amplitude (Fig. 4A). In contrast, when the glucose concentration was increased simultaneously with the lengthening of the forced $[Ca^{2+}]_i$ oscillations, the amplitude of the insulin secretion pulses was clearly increased (Fig. 4B).

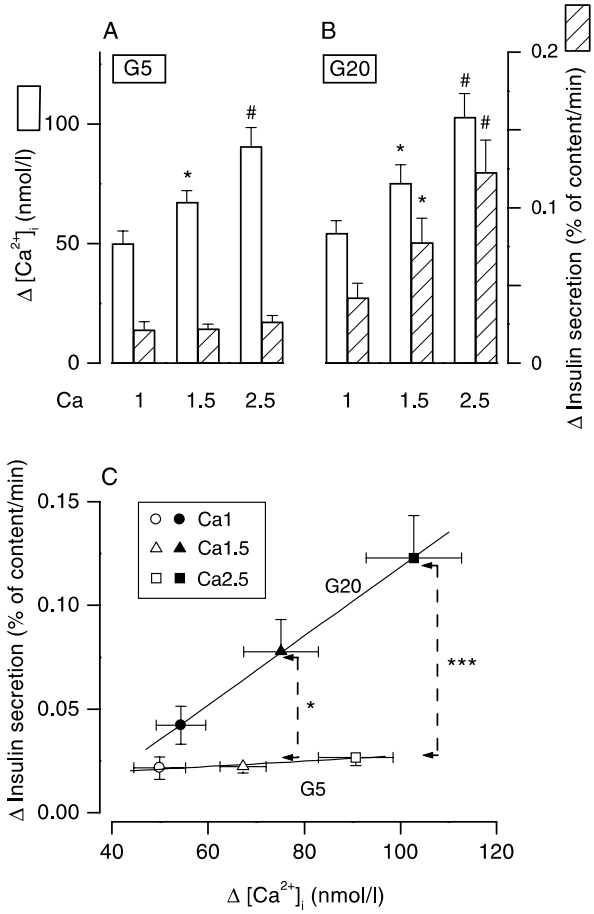

Fig. 3. Quantification of $[Ca^{2+}]_i$ and insulin secretion changes in the experiments of Fig. 2 and in two similar experiments in which extracellular $CaCl_2$ was changed in reverse order. Δ $[Ca^{2+}]_i$ and Δ insulin were calculated as in Fig. 1. A,B: $^*P < 0.05$ or less between 1.5 and 1 mmol/l $CaCl_2$ and $^\#P < 0.01$ between 2.5 and 1.5 mmol/l $CaCl_2$. C: Relationship between Δ insulin secretion and Δ $[Ca^{2+}]_i$ in 5 or 20 mmol/l glucose (G5 or G20) and 1, 1.5 or 2.5 mmol/l $CaCl_2$ as indicated. $^*P < 0.05$, $^{***}P < 0.001$. Values are means ± S.E.M. for six islets.

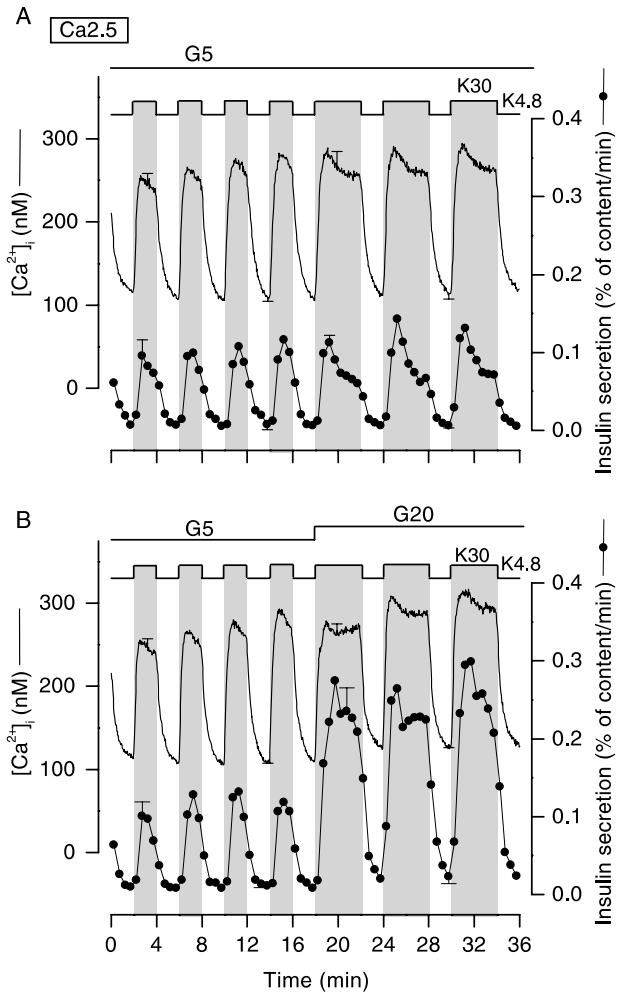

Fig. 4. Time and amplitude modulation of insulin secretion triggered by $[Ca^{2+}]_i$ pulses in β-cells. $[Ca^{2+}]_i$ and insulin secretion were measured in the same single islets. The K^+ concentration was increased from 4.8 to 30 mmol/l for 2 min every 4 min, or for 4 min every 6 min as indicated. The concentration of glucose (G) was either kept at 5 mmol/l throughout (A) or increased from 5 to 20 mmol/l together with the lengthening of the K^+ pulses (B). Values are means ± S.E.M. for six islets.

4. Discussion

The present study shows that the amplifying pathway of glucose control of insulin secretion is operative when $[Ca^{2+}]_i$ is forced to change repetitively, in a way resembling the slow $[Ca^{2+}]_i$ oscillations occurring in glucose-stimulated mouse islets. Thus, glucose markedly increased the amplitude of the insulin secretion pulses although the size of the triggering $[Ca^{2+}]_i$ pulses barely changed. Previous experiments have established that an elevation of $[Ca^{2+}]_i$ in β-cells is necessary for the amplifying action of glucose to manifest itself [1]. The novel information is that this $[Ca^{2+}]_i$ elevation does not have to be continuous, as was the case in all previously used paradigms. The fact that the amplifying pathway increases insulin secretion when $[Ca^{2+}]_i$ oscillates validates previous extrapolations on the role of this pathway under physiological conditions, such as the second phase of glucose stimulation in mouse and human islets. It is noteworthy that the selected rhythm of imposed $[Ca^{2+}]_i$ oscillations was similar to the frequency of the major spontaneous slow oscillations of $[Ca^{2+}]_i$ in glucose-stimulated islets [6,17] and of the oscillations of insulin secretion in vitro and in vivo [6,14].

Using controlled stepwise, sustained elevations of $[Ca^{2+}]_i$ in mouse islets, we previously showed that the amplifying action of glucose on insulin secretion increases with $[Ca^{2+}]_i$ [23]. A similar phenomenon was observed here for imposed $[Ca^{2+}]_i$ pulses. Thus, increasing the amplitude of $[Ca^{2+}]_i$ pulses at low glucose or increasing the glucose concentration at modestly elevated $[Ca^{2+}]_i$ had little impact on insulin secretion as compared with the marked amplification of insulin secretion that accompanied similar changes at higher glucose or $[Ca^{2+}]_i$.

In mouse islets studied in vitro, when ambient glucose is increased from a moderately stimulatory to a strongly stimulatory concentration, the phases of depolarization increase in duration, and so do the corresponding oscillations of $[Ca^{2+}]_i$ [6,22]. Importantly, although the amplitude of these $[Ca^{2+}]_i$ oscillations does not change, that of the accompanying insulin pulses increases [30]. In single perifused mouse islets [26], in batches of perifused rat islets [27] and in normal human subjects [14], a rise in the glucose concentration also essentially increases the amplitude of the slower pulses of insulin secretion. There is thus an apparent discrepancy between a consistent increase of pulsatile secretion and no change in the amplitude of $[Ca^{2+}]_i$ oscillations in islets. Two explanations have tentatively been proposed. First the increase in glucose might recruit more and more β-cells into an active state [26]. However, the homogeneity of $[Ca^{2+}]_i$ changes in different regions of the islets [6,19,21,30] and the evidence that recruitment to produce a Ca^{2+} response is virtually complete at 10 mmol/l glucose [31] do not support the hypothesis. At least, if recruitment into a secretory state is involved, the implicated mechanism is not only the $[Ca^{2+}]_i$ rise [31]. Second, the lengthening of the period of $[Ca^{2+}]_i$ elevation has been suggested to cause not only a longer, but also a larger phase of insulin secretion [18]. The present study refutes this interpretation. Mere lengthening of the $[Ca^{2+}]_i$ pulse did not influence the amplitude of the insulin pulses, whereas the rise in glucose concentration increased it. The amplifying pathway thus seems to underlie this increase in amplitude.

We still do not know why the β-cell response to glucose is oscillatory. One widely held view is that pulsatile insulin secretion optimizes the peripheral action of the hormone [12–14]. Alternatively, oscillations of different signals, including $[Ca^{2+}]_i$, may be important for β-cell functions other than secretion itself [6,32]. The present study suggests a possible functional advantage of $[Ca^{2+}]_i$ oscillations. An intermittent larger rise of $[Ca^{2+}]_i$ as that occurring at the peak of each $[Ca^{2+}]_i$ oscillation may reach a concentration zone where the amplifying action of glucose is effective, whereas a lesser, sustained $[Ca^{2+}]_i$ rise would not. Anyhow, the coexistence of a time-dependent modulation via the triggering pathway and an amplitude-dependent modulation via the amplifying pathway ensures tight regulation of insulin secretion.

Acknowledgements: This work was supported by grants from the Fonds National de la Recherche Scientifique, the General Direction of Scientific Research of the French Community of Belgium, and the Federal Office for Scientific, Technical, and Cultural Affairs from Belgium. We thank F. Knockaert for technical assistance and V. Lebec for editorial help.

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