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Disorganization of cytoplasmic Ca^{2+} oscillations and pulsatile insulin secretion in islets from *ob/ob* mice

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Abstract

Aims/hypothesis. In normal mouse islets, glucose induces synchronous cytoplasmic $[Ca^{2+}]_i$ oscillations in beta cells and pulses of insulin secretion. We investigated whether this fine regulation of islet function is preserved in hyperglycaemic and hyperinsulinaemic *ob*/*ob* mice.
Methods. Intact islets from *ob*/*ob* mice and their lean littermates were used after overnight culture for measurement of $[Ca^{2+}]_i$ and insulin secretion.
Results. We observed three types of $[Ca^{2+}]_i$ responses during stimulation by 9 to 12 mmol/l of glucose: sustained increase, rapid oscillations and slow (or mixed) oscillations. They occurred in 8, 18 and 74% of lean islets and 9, 0 and 91% of *ob*/*ob* islets, respectively. Subtle desynchronisation of $[Ca^{2+}]_i$ oscillations between regions occurred in 11% of lean islets. In *ob*/*ob* islets, desynchronisation was frequent (66–82% depending on conditions) and prominent: oscillations were out of phase in different regions because of distinct periods and shapes. Only small *ob*/*ob* islets were well synchronised, but sizes of synchronised lean and desynchronised *ob*/*ob* islets were markedly overlapped. The occurrence of desynchronisation in clusters of 5 to 50 islet cells from *ob*/*ob* mice and not from lean mice further indicates that islet hypertrophy is not the only causal factor. In both types of islets, synchronous $[Ca^{2+}]_i$ oscillations were accompanied by oscillations of insulin secretion. In poorly synchronised *ob*/*ob* islets, secretion was irregular but followed the pattern of the global $[Ca^{2+}]_i$ changes.
Conclusions/interpretation. The regularity of glucose-induced $[Ca^{2+}]_i$ oscillations is disrupted in islets from *ob*/*ob* mice and this desynchronisation perturbs the pulsatility of insulin secretion. A similar mechanism could contribute to the irregularity of insulin oscillations in Type II (non-insulin-dependent) diabetes mellitus. [Diabetologia (2002) 45:1154–1163]

Keywords Insulin secretion, pancreatic islet, beta cell, cytosolic Ca^{2+}, pulsatility, *ob*/*ob* mouse.

Plasma insulin concentration regularly oscillates in normal subjects [1, 2, 3]. These oscillations are perturbed in patients with overt Type II (non-insulin-dependent) diabetes mellitus, obesity or isolated insulin-resistance [4, 5, 6, 7]. It has also been reported that exogenous insulin is more efficient when administered in a pulsatile rather than a continuous manner [8, 9]. All these observations suggest that the oscillations of plasma insulin are important for optimal glucose homeostasis. Although the origin of these oscillations is still not completely understood, there is good evidence that they result from pulsatile insulin secretion [3, 10, 11, 12].

Several aspects of beta-cell function are characterised by oscillations; they include metabolism [13, 14, 15, 16], changes in membrane potential [17, 18, 19] and changes in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) [20, 21, 22, 23]. During glucose stimulation good temporal correlations have been found between $[Ca^{2+}]_i$ oscillations in beta cells and insulin secretion measured

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Abbreviations: $[Ca^{2+}]_i$, Cytoplasmic free Ca^{2+} concentration.

simultaneously from the same islet [24, 25, 26, 27, 28]. It could thus be hypothesised that the perturbations of pulsatile insulin secretion are secondary to a loss of regularity of $[Ca^{2+}]_i$ oscillations in the islet. We now report such a phenomenon in islets from chronically hyperinsulinaemic and hyperglycaemic *ob*/*ob* mice.

Materials and methods

Preparation and solutions. The control medium used for islet isolation was a bicarbonate-buffered solution containing (in mmol/l) NaCl 120, KCl 4.8, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 24, glucose 10, 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 was used for the experiments.

The Swedish *ob*/*ob* mice are traditionally used as islet donors at an age between 8 and 13 months [26, 29, 30, 31]. The experiments were thus carried out with 23 non-inbred female obese *ob*/*ob* mice (7.5–14 months, mean age 10.3 months) and 22 lean littermates (7.5–14 months, mean age 10.1 months) from the Umea colony. After their transfer from Umea to Brussels, the mice were allowed to adapt to their new environment for at least 2 weeks. The animals were killed by decapitation in the fed state. Blood glucose was measured with a glucometer (Bayer, Zurich, Switzerland) and plasma was saved for insulin assay. After aseptic digestion of the pancreas with collagenase, isolated islets were selected by hand-picking [32]. The islets were then cultured for 1 day in RPMI-1640 medium (Gibco, Paisley, UK) containing 5.5 or 10 mmol/l glucose, 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin.

Some experiments were also carried out with clusters of islet cells obtained by incubating the islets for 5 min in Ca^{2+}-free medium followed by dissociation in RPMI medium by gentle pipetting through a siliconised glass pipette. The clusters were then cultured for 1 day on 22 mm^2 circular glass coverslips placed in petri dishes containing RPMI medium with 10 mmol/l glucose [33].

The study was approved by the commission d'éthique pour l'expérimentation animale (project ENDO/98/01) of the University of Louvain faculty of medicine.

Measurement of $[Ca^{2+}]_i$ and insulin secretion. The system has previously been described [22, 32]. Cultured islets were loaded with fura-PE3 during 2 h of incubation at 37°C in control medium containing 2 µmol/l fura-PE3 acetoxymethylester and the same glucose concentration (5.5 or 10 mmol/l) as that during the culture. When insulin secretion was to be measured at the same time as $[Ca^{2+}]_i$ one loaded islet was transferred into a perifusion chamber with a bottom made of a glass coverslip and mounted on the stage of a microscope. The islet was held in place by gentle suction with a micropipette. The preparation was perifused at a flow rate of 1.8 ml/min and the medium, that contained the same glucose concentration (9 or 12 mmol/l) throughout, was collected just downstream of the islet at 30 s intervals. The temperature within the chamber was 37°C. Approximately 10 min after the transfer of the islet into the chamber, $[Ca^{2+}]_i$ was measured by dual wavelength (340 and 380 nm) excitation spectrofluorimetry, using a CCD camera to capture images (510 nm) at a resolution of one measured concentration every 3.08 or 1.48 s permitting experiments of 40 and 20 min respectively. From the ratio of fluorescence at 340 and 380 nm, the concentration of $[Ca^{2+}]_i$ was calculated by comparison with a calibration curve [22]. This calculation was done for the whole islet to obtain the global response and for subregions of the islet to assess the synchrony of $[Ca^{2+}]_i$ changes throughout the islet. Insulin was measured in duplicates, in 400 µl aliquots of the effluent fractions. The characteristics of the radioimmunoassay, using rat insulin as a standard, have been described [32]. When insulin secretion was not measured, two islets were studied at a time, and the perifusion medium was not collected. At the end of the experiment, the size of the islets was estimated from their largest and smallest diameters measured on the screen of the recording system. The volume was then calculated assuming an ovoid shape.

Clusters of islet cells attached to the coverslip were loaded with fura-PE3 during 2 h. The coverslip was then transferred to the perifusion chamber in which it serves as a bottom. $[Ca^{2+}]_i$ was measured as described above for the islets, at a resolution of one measured concentration every 2.68 s. Insulin secretion was not measured in these experiments. At the end, the preparation which was still on the stage of the microscope was stained with bisbenzimide to permit counting of the nuclei within the studied clusters [33].

Presentation of results. $[Ca^{2+}]_i$ changes are illustrated by representative traces, with indication of the incidence of each type of response. The traces usually show the global response of the whole islet and the response of several regions of the islet. Other results are shown as means ± SEM, and the statistical significance of their differences was assessed by unpaired Student's *t* test or ANOVA followed by Newman-Keuls test as appropriate. Islet volumes were compared by non-parametric Kruskal-Wallis test, and proportions by Fisher's exact test. Differences were considered significant with a p value of less than 0.05.

Results

Characteristics of the mice and the islets. The body weight, blood glucose and plasma insulin concentrations were: 26.7±0.7 g, 6.7±0.1 mmol/l, 1.4±0.2 ng/ml, respectively, for lean mice (n=22), and 69.5±1.4 g (p<0.001), 9.2±0.6 mmol/l (p<0.001), 295±41 ng/ml (p<0.001), respectively, for *ob*/*ob* mice (n=23). The *ob*/*ob* mice were thus very obese, slightly hyperglycaemic and markedly hyperinsulinaemic, as previously reported for this Swedish strain [34, 35].

The majority of islets from *ob*/*ob* mice are much larger than those of lean mice [36]. However, these very big islets were deliberately not taken for the experiments because they showed a high incidence of central necrosis after 1 day of culture. The mean volume of the tested islets from *ob*/*ob* mice was about one third larger than that of the tested islets from lean mice (in $mm^3 \cdot 10^{-3}$: 2.99±0.16, n=107 vs 2.15±0.09, p<0.001 n=110).

General characteristics of $[Ca^{2+}]_i$ responses of the islets. When *ob*/*ob* and lean mouse islets were perifused with a nonstimulatory glucose concentration (3 mmol/l), basal $[Ca^{2+}]_i$ was stable at about 100 nmol/l. The $[Ca^{2+}]_i$ responses observed during continuous perifusion with a medium containing 9 or 12 mmol/l glucose had similar characteristics at the two glucose concentrations and will thus subsequently be described without distinction. Three types of $[Ca^{2+}]_i$ responses were identified (Table 1). A steady $[Ca^{2+}]_i$ increase (212±13 nmol/l, n=13) without oscil-

Table 1. Types of $[Ca^{2+}]_i$ responses in lean and *ob*/*ob* mouse islets

	Culture in 10 mmol/l glucose		Culture in 5.5 mmol/l glucose	
	Lean	*ob*/*ob*	Lean	*ob*/*ob*
Total number of islets	83	67	27	40
No $[Ca^{2+}]_i$ oscillations	7	6	0	0
$[Ca^{2+}]_i$ oscillations	76	61	27	40
Only rapid	15	0	0	0
Slow or mixed	61 (=100%)	61 (=100%)	27 (=100%)	40 (=100%)
Synchronous	53 (87%)	21 (34%)[a]	25 (93%)	7 (18%)[a]
Asynchronous	8 (13%)	40 (66%)[a]	2 (7%)	33 (82%)[a]

[a] $p<0.001$ vs islets from lean mice cultured at the same glucose concentration

After 1 day of culture in the presence of the indicated glucose concentration, the islets were perifused with 9 or 12 mmol/l glucose during the whole experiments

lations occurred in a minority (9%) of lean and *ob*/*ob* islets after culture in 10 mmol/l glucose whereas this type of response was not seen after culture in 5.5 mmol/l glucose. The second type of response, consisted of continuous fast (2–6 per min) $[Ca^{2+}]_i$ oscillations with an amplitude of 30–60 nmol/l (Fig. 1). This was only seen in 15 lean islets (18%) cultured in 10 mmol/l glucose. In 7 out of these 15 islets the image resolution was insufficient (less than six data points per oscillation) to permit reliable assessment of the synchrony of these fast $[Ca^{2+}]_i$ transients between regions of the islets. The resolution was sufficient to carry out the analysis in the other eight islets, and the rapid $[Ca^{2+}]_i$ oscillations were found to be synchronous throughout five of these islets (Fig. 1). Although the shape and amplitude of the individual $[Ca^{2+}]_i$ transients varied between regions, virtually all of them were detectable in the four regions of the islet. The frequency of six per min (Fig. 1) was the highest at which synchrony could be found between rapid $[Ca^{2+}]$ transients.

The third type of response was characterised by slow $[Ca^{2+}]_i$ oscillations (0.2–1 per min; amplitude of 50–200 nmol/l) that occurred either alone or together with rapid oscillations (mixed pattern). These slow or mixed oscillations characterised 73% (61/83) of the lean islets and 91% (61/67) of the *ob*/*ob* islets after culture in 10 mmol/l glucose and all lean and *ob*/*ob* islets after culture in 5.5 mmol/l glucose (Table 1). They were analysed in greater detail to evaluate their degree of synchronisation throughout the islet and their temporal correlation with insulin secretion. Note, however, that insulin secretion was not measured in all experiments.

Glucose-induced $[Ca^{2+}]_i$ and insulin oscillations in islets from lean mice. Figure 2 shows one example of mixed $[Ca^{2+}]_i$ oscillations induced by glucose in an islet from a lean mouse. The upper panel (A) shows the signal integrated over the whole islet, and the temporal correlation between these global $[Ca^{2+}]_i$ oscilla-

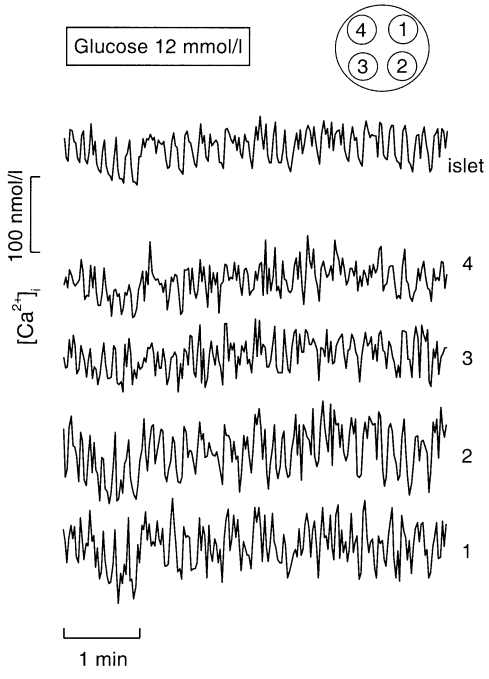

Fig. 1. Synchrony of the fast $[Ca^{2+}]_i$ oscillations induced by 12 mmol/l glucose in an islet from a lean mouse. Only 5 min of a 20 min experiment are shown. Forty data points were collected every min. The upper trace shows the global response of the islet, and the lower traces show the response of the four indicated regions

tions and oscillations of insulin secretion. The lower panel (Fig. 2B) decomposes the global $[Ca^{2+}]_i$ response into signals recorded over seven sub-regions of the islet. The synchrony of the response was good except during the fifth oscillation that was duplicated and slightly smaller in regions 1, 2, 6 and 7, resulting in a broader $[Ca^{2+}]_i$ peak in the global response. This partial and minimal loss of synchrony occurred only once during the 40 min of the experiment and this islet was considered synchronised. It is impressive that this subtle change in the shape of the $[Ca^{2+}]_i$ oscillation had an impact on the pattern of insulin secretion (Fig. 2A).

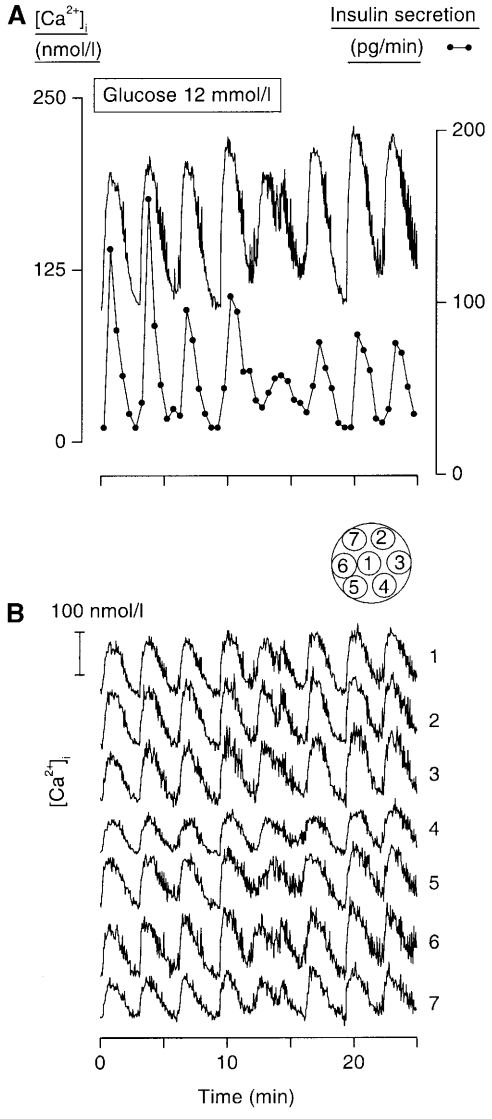

Fig. 2A, B. Synchronous mixed $[Ca^{2+}]_i$ oscillations and their synchrony with insulin secretion in a lean mouse islet stimulated by 12 mmol/l glucose. The trace in (**A**) shows the global response of the islet, and the traces in (**B**) show the response in the seven indicated regions. For insulin secretion measurement (**A**), the effluent was collected every 30 s

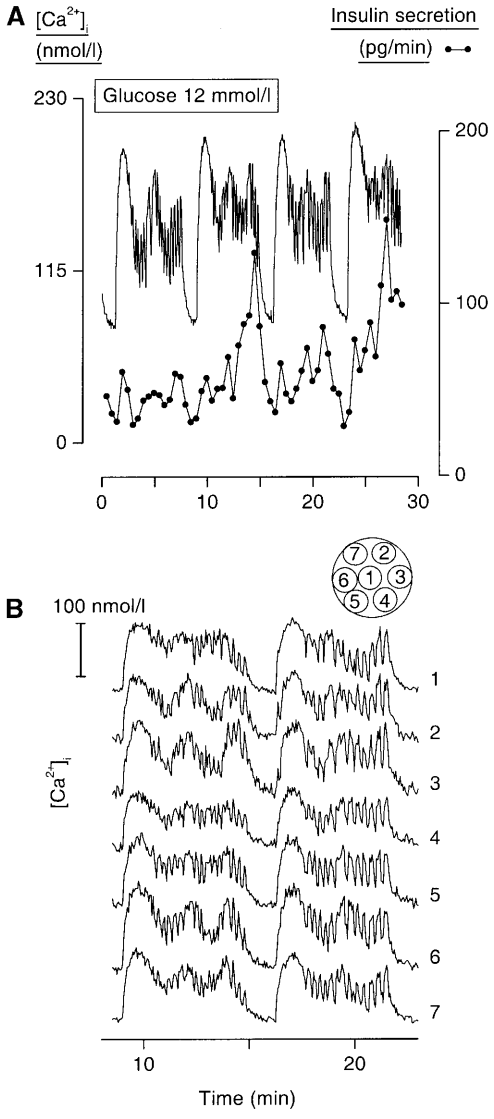

Fig. 3A, B. Synchronous mixed $[Ca^{2+}]_i$ oscillations and their synchrony with insulin secretion in an *ob*/*ob* mouse islet stimulated by 12 mmol/l glucose. The trace in (**A**) shows the global response of the islet, and the traces in (**B**) show the response in the seven indicated regions. For insulin secretion measurement (**A**), the effluent was collected every 30 s

Islets were categorised as asynchronised when one region showed stable $[Ca^{2+}]_i$ (low or high) besides others showing synchronous $[Ca^{2+}]_i$ oscillations, or when several oscillations were not in phase in two or more regions. The incidence of incomplete synchronisation of lean mouse islets was similar after culture in 10 and 5.5 mmol/l glucose (Table 1). For both groups together, it amounted to 11% (10/88 islets).

Glucose-induced $[Ca^{2+}]_i$ and insulin oscillations in islets from ob/ob mice. Only one third of *ob*/*ob* mouse islets cultured in 10 mmol/l glucose showed synchronous slow or mixed $[Ca^{2+}]_i$ oscillations during glucose stimulation, which is a much lower proportion than for lean mouse islets (Table 1). This proportion was even lower in *ob*/*ob* islets cultured in 5.5 mmol/l glucose (18%). Figure 3 illustrates the response of one of these islets. The synchrony was good throughout the islet for the slow oscillations and most of the rapid ones (Fig. 3B). Note that the rapid oscillations were present in all regions.

Figure 3A also shows the temporal correlation between $[Ca^{2+}]_i$ and insulin secretion changes. The initial peak of each slow $[Ca^{2+}]_i$ oscillation was paralleled by a peak of secretion, and the subsequent rapid oscillations were accompanied by a progressively increasing secretory response. As the sampling rate for insulin assay was lower than the frequency of the rapid $[Ca^{2+}]_i$ oscillations, it was not possible to determine if also these oscillations cause pulsatile insulin secretion.

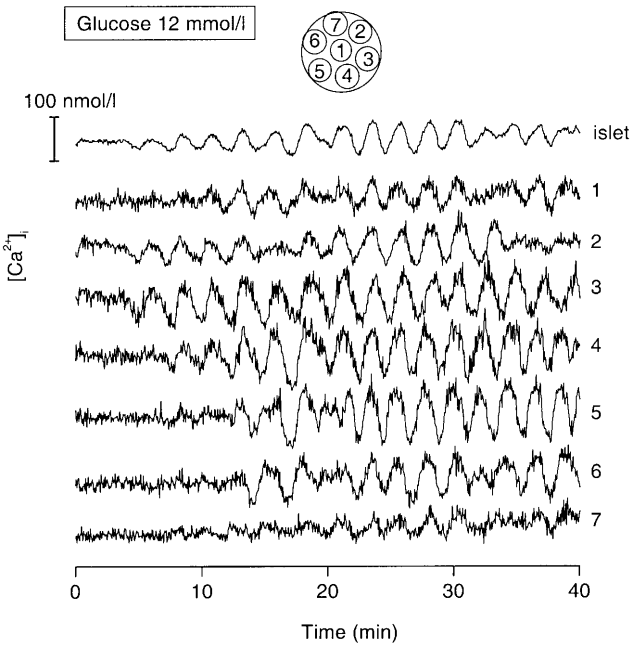

Fig. 4. Asynchronous $[Ca^{2+}]_i$ oscillations in an *ob*/*ob* mouse islet stimulated by 12 mmol/l glucose. The upper trace shows the global response of the islet, and the lower traces show the response in the seven indicated regions

In more than two-thirds of *ob*/*ob* islets, glucose-induced slow or mixed $[Ca^{2+}]_i$ oscillations were poorly synchronised. The pattern shown in Fig. 4 illustrates several characteristics of this desynchronisation. During the first 5 min, $[Ca^{2+}]_i$ was steadily increased (195 nmol/l). Then, oscillations appeared with an increasing (until 28 min) and eventually decreasing amplitude. Analysis of the signal in seven regions of the islet explains these changes. At the beginning, the rapid, small and asynchronous variations of $[Ca^{2+}]_i$ occurring in the different regions cancelled out, so that the signal integrated over the whole islet looked rather stable. $[Ca^{2+}]_i$ then started to oscillate in phase in regions 2, 3 and 4, which resulted in small oscillations in the global response because regions 1, 5, 6 and 7 still maintained a stable increase of $[Ca^{2+}]_i$. A few minutes later, synchronous oscillations of different amplitude also appeared in these regions. In the whole islet the amplitude of $[Ca^{2+}]_i$ oscillations reached a maximum between 22 and 28 min, when the synchrony was best between regions, and then decreased again. Note that, even when the islet seemed to be well synchronised, a time lag in the appearance of the oscillations persisted. In other islets, $[Ca^{2+}]_i$ constantly remained stable in some regions and oscillated in others.

Another type of desynchronisation is shown in Fig. 5. In this islet, all regions showed $[Ca^{2+}]_i$ oscillations during the 40 min of experiment but the global response was changing because the regularity and the frequency of the oscillations were variable between regions. Occasionally, the time shifts between oscillations in adjacent regions resulted in the appearance of waves of increased $[Ca^{2+}]_i$. Around 4 min, a wave crossed the islet linearly from the upper left to the lower right direction, i.e. perpendicularly to the flow of the perifusion medium (Fig. 5A). Around 31 min, another wave crossed the islet from the lower left to the upper right direction, i.e. opposite to the flow (Fig. 5C). Just before, however, around 29 min, no $[Ca^{2+}]_i$ wave was detected. The increase of $[Ca^{2+}]_i$ began at the two poles of the islet, and so did the decrease in $[Ca^{2+}]_i$ (Fig. 5B). When a wave was visible, its speed of progression was slow (less than 5 µm/s). However, these waves do not seem to reflect intercellular Ca^{2+} propagation but to result from a fortuitously adequate time shift between asynchronous oscillations in different regions. This could explain why they progress in different directions within the same islet. Importantly, during the period 32–38 min, the global $[Ca^{2+}]_i$ signal did not oscillate although distinct oscillations were present in all regions, because these oscillations were completely desynchronised.

Figure 6 shows, with another time scale, the global $[Ca^{2+}]_i$ changes occurring in the same islet, and the correlation between these changes and insulin secretion. Whenever a distinct peak of $[Ca^{2+}]_i$ occurred, a pulse of insulin secretion was triggered, whereas the secretion rate was more stable when $[Ca^{2+}]_i$ did not clearly oscillate in the whole islet (period 32–38 min). The temporal pattern of insulin secretion thus followed the irregularities of the $[Ca^{2+}]_i$ pattern.

Influence of islet size on synchronisation of $[Ca^{2+}]_i$ oscillations. Figure 7 shows the volume distribution of lean and *ob*/*ob* mouse islets with synchronous and asynchronous $[Ca^{2+}]_i$ oscillations after culture in 10 or 5.5 mmol/l glucose. Regardless of the glucose concentration during culture, asynchronous *ob*/*ob* islets were larger ($p<0.001$) than synchronous lean and *ob*/*ob* islets. Asynchronous lean islets were also larger ($p<0.05$) than synchronous ones when the two groups (cultured in 10 and 5.5 mmol/l glucose) were pooled but not when they were considered individually. Although these comparisons indicate that asynchrony could be linked to islet size, the overlap between the different size groups is marked. We therefore compared lean and *ob*/*ob* islets grouped in three classes of size (encompassing 90% of lean islets): 1 to 2, 2 to 3 and 3 to 4 $mm^3 \cdot 10^{-3}$. The prevalence of desynchronisation was 7, 12 and 22% respectively, for lean islets, and 45, 75 and 100% respectively, for *ob*/*ob* islets. Thus, the proportion of asynchronous responses increased with the islet size and within each class of size was much higher ($p<0.001$) in *ob*/*ob* than lean islets although mean sizes were similar (e.g for class 2–3 $mm^3 \cdot 10^{-3}$: 2.5±0.06 in both groups, with $n=24$ and $n=25$).

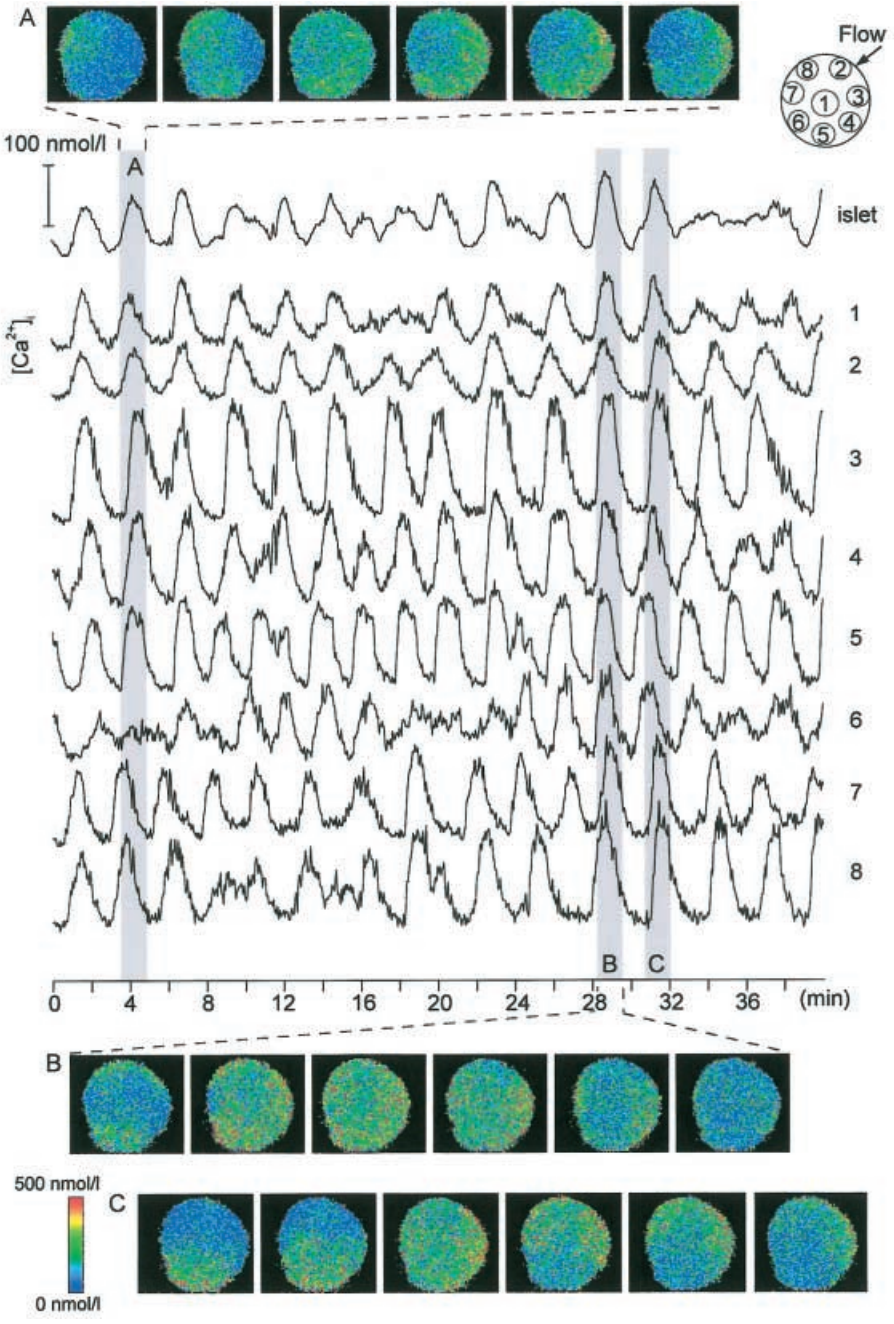

Fig. 5A–C. Asynchronous $[Ca^{2+}]_i$ oscillations and $[Ca^{2+}]_i$ waves in an *ob*/*ob* mouse islet stimulated by 12 mmol/l glucose. The upper trace shows the global response of the islet, and the lower traces show the response in the eight indicated regions. The series of pseudo color images correspond to the periods indicated by the *shaded areas*. **A** and **C** illustrate $[Ca^{2+}]_i$ waves crossing the islet linearly, but in different directions. **B** Shows an oscillation of $[Ca^{2+}]_i$ without wave. The intervals between successive images were 15.4 s

As already mentioned, the very big islets of *ob*/*ob* mice were not used for the comparisons shown. In the few experiments (n=7) carried out with *ob*/*ob* islets at least fivefold bigger than lean islets (volume range: 12–28 $mm^3 \cdot 10^{-3}$), desynchronisation of $[Ca^{2+}]_i$ oscillations was consistently observed.

Glucose-induced $[Ca^{2+}]_i$ oscillations in clusters of islet cells. During continuous perifusion with 12 mmol/l glucose, clusters of 5–50 islet cells from lean and *ob*/*ob* mice showed slow $[Ca^{2+}]$ oscillations at a similar mean frequency of 0.23±0.01 per min, with occasionally a mixed pattern. These characteristics are in agreement with those previously reported for islet cell clusters from lean white [33, 37] and *ob*/*ob* mice [38]. In none of the 33 clusters prepared from four lean mice was the $[Ca^{2+}]_i$ signal asynchronous between cells. In contrast, 7/38 clusters (p<0.001) prepared from four *ob*/*ob* mice were asynchronous (Fig. 8). In a cluster of five cells, two cells, which were initially synchronised with the other three, transiently stopped producing $[Ca^{2+}]_i$ oscillations (Fig. 8A). In a larger cluster of 45 cells, $[Ca^{2+}]_i$ oscillations occurring in re-

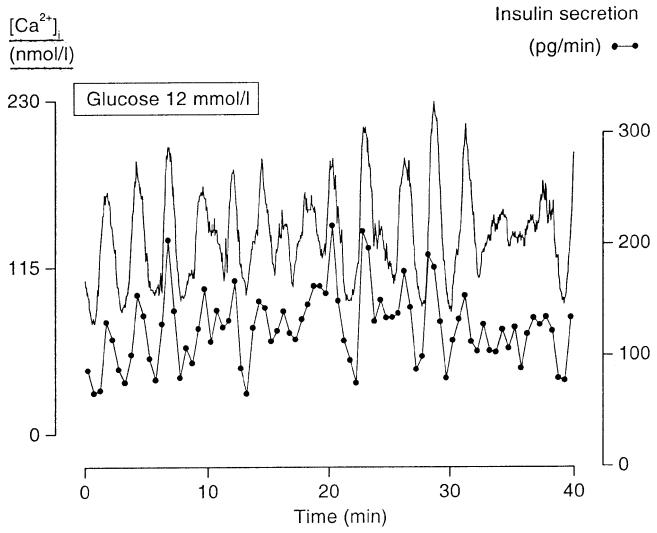

Fig. 6. Temporal correlation between the poorly synchronised $[Ca^{2+}]_i$ oscillations and insulin secretion in an *ob*/*ob* mouse islet stimulated by 12 mmol/l glucose. Same islet as that shown in Fig. 5

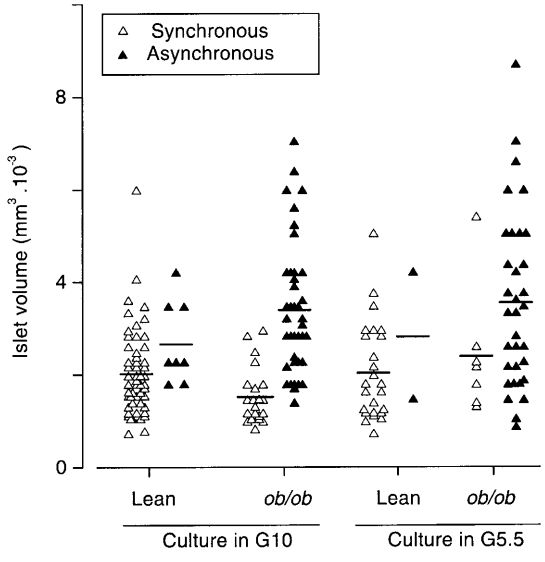

Fig. 7. Size distribution of the 189 lean and *ob*/*ob* mouse islets showing synchronous or asynchronous $[Ca^{2+}]_i$ oscillations (of the slow or mixed type) in responses to 9 or 12 mmol/l glucose. The islets were cultured in 10 or 5.5 mmol/l glucose as indicated. The *horizontal bars* indicate the mean in each group

gion 2 almost consistently showed a slower ascending phase than in region 1 (Fig. 8B). The asynchrony in clusters was less frequently detected (19 vs 66% for preparations cultured in 10 mmol/l glucose) and was not so spectacular as in intact islets from *ob*/*ob* mice. It was not directly related to cluster size: 33, 13 and 18% of the clusters containing 5–14, 15–30 and 31–50 cells, respectively.

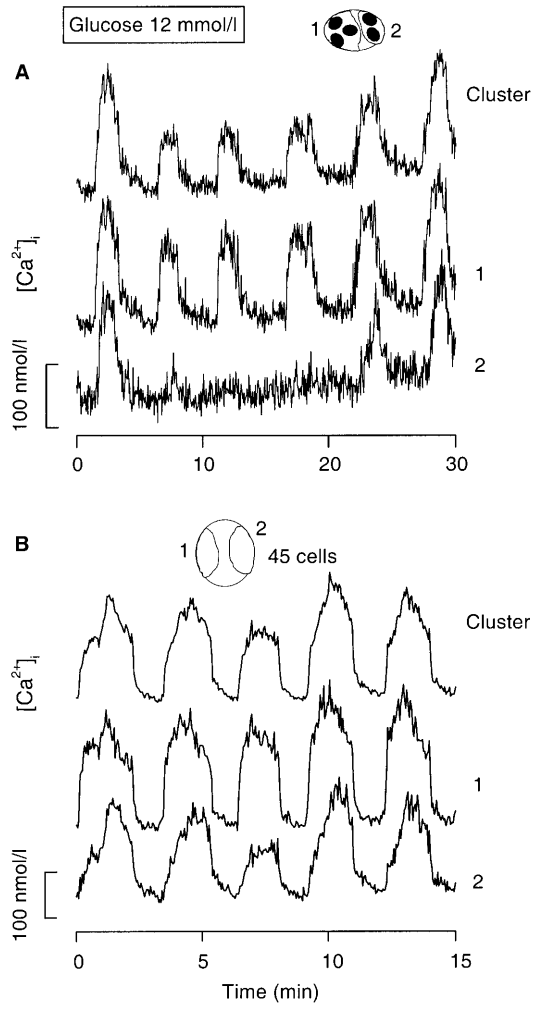

Fig. 8A, B. Asynchronous $[Ca^{2+}]_i$ oscillations in islet cell clusters from *ob*/*ob* mice stimulated by 12 mmol/l glucose. In each panel the upper trace shows the global response of the cluster, and the lower two traces show the response in the two *shaded regions*. **A** Cluster of five cells with indicated position of the nuclei. **B** Cluster of 45 cells

Discussion

The experiments were carried out with 8 to 14 month old *ob*/*ob* mice because these Swedish obese mice are traditionally used as islet donors at this advanced age [26, 29, 30, 31]. For comparison, we also studied islets from their lean littermates at a similar age. The results show that glucose-induced $[Ca^{2+}]_i$ oscillations are much more often asynchronous in islets from *ob*/*ob* than lean mice.

Measuring $[Ca^{2+}]_i$ in whole islets is complicated by uneven loading of the fluorescent dye and the presence of several layers of cells [31, 39]. However, we are confident that our results are representative of the changes occurring in the core of beta cells for two reasons: firstly, the recorded $[Ca^{2+}]_i$ changes correlate well with membrane potential changes known to occur in beta cells [21, 22, 23] and are similar to those observed in beta cells within islets using confocal

microscopy [39]; secondly, oscillations of insulin secretion parallel the oscillations of $[Ca^{2+}]_i$ recorded during glucose stimulation [24, 25]. No similar temporal correlation could exist between secretion, a response of all beta cells of the islet, and a $[Ca^{2+}]_i$ signal restricted to a superficial layer of cells if these were not representative of the whole beta cell population.

It is widely agreed that glucose-induced $[Ca^{2+}]_i$ oscillations occur synchronously in all regions of intact islets from normal mice [21, 22, 25, 39]. An excellent synchrony also characterises $[Ca^{2+}]_i$ responses to glucose in all beta cells within monolayer clusters cultured overnight [33, 37]. Even with high time resolution, no marked or consistent desynchronisation between normal beta cells is detected; only small phase lags of 1 to 2 s can sometimes be seen between oscillations in different islet regions [21]. Waves of $[Ca^{2+}]_i$ propagating alongside the periphery or across intact mouse islets have been described by two groups [40, 41]. These waves were said to occur, at times, after 3 days of culture [40] or almost consistently after 1 to 2 weeks of culture [41]. Waves of $[Ca^{2+}]_i$ propagating in the direction of the stream within and between clusters of beta cells have been observed after culture for 6 to 15 days [40]. In all these experiments, the speed of propagation of the Ca^{2+} signal was between 30 and 100 μm/s [41]. Overall, the published results indicate that synchrony prevails and $[Ca^{2+}]_i$ waves are absent in glucose-stimulated islets from normal mice, but that asynchrony and waves can appear after several days of culture. The synchrony of $[Ca^{2+}]_i$ oscillations and the absence of $[Ca^{2+}]_i$ waves in islets from lean mice, studied here after overnight culture, are in agreement with the above conclusion.

Single beta cells from *ob*/*ob* mice have often been used as a model to study $[Ca^{2+}]$ handling by insulin-secreting cells [42]. With a few exceptions [43], the characteristics of their responses were comparable to those obtained by others in single beta cells from lean mice [12]. On the other hand, only a few $[Ca^{2+}]_i$ measurements have been carried out in clusters of cells [38] or intact islets from *ob*/*ob* mice [26, 31]. Propagation of $[Ca^{2+}]_i$ waves through a freshly prepared, well synchronised cluster of 9 *ob*/*ob* beta cells has been described [38]. However, the relative prevalence of synchronous and asynchronous $[Ca^{2+}]_i$ oscillations in glucose-stimulated *ob*/*ob* mouse islets has not been reported [26, 31]. It has only been mentioned that some islets comprise regions in which $[Ca^{2+}]_i$ oscillations are either slow or rapid, and the sum of the two patterns was suggested to give rise to the mixed $[Ca^{2+}]_i$ oscillations in whole islets [31]. Our interpretation is that these different responses in distinct regions reflect a lack of synchronisation. In our study, apparent waves of increased $[Ca^{2+}]_i$ were observed in several *ob*/*ob* islets. However, we do not believe that they reflect propagation of the signal from a pacemaker region for several reasons: these waves were inconsistent, progressed slowly (<5 μm/s) and followed variable directions. They seem to result from coincidental phase shifts of $[Ca^{2+}]_i$ oscillations in neighbouring regions. True, rapid $[Ca^{2+}]_i$ waves could exist but the time resolution of our system would not permit their detection.

The oscillations of $[Ca^{2+}]_i$ occurring in glucose-stimulated beta cells primarily depend on an intermittent influx of Ca^{2+} through voltage-dependent Ca^{2+} channels activated by cyclic depolarisations of the plasma membrane [21, 22, 23]. The synchrony of $[Ca^{2+}]_i$ oscillations in normal mouse islets is attributed to the synchrony of the membrane potential changes in all cells of the islets. Whether the coupling is strictly electrical, through gap junctions, or involves extracellular signals, is still disputed [44]. Recordings with intracellular electrodes have shown that glucose induces oscillations of the membrane potential with bursts of action potentials in *ob*/*ob* beta cells within intact islets, but have identified several differences with the electrical pattern in normal mouse islets [45]. However, the authors emphasised that their observations, made with *ob*/*ob* mice from the Aston colony, might not be applicable to *ob*/*ob* mice of the Umea colony. These have not been used for intracellular recordings, and the degree of electrical coupling of their beta cells in situ is therefore not known. Our results showing poor synchronisation of the $[Ca^{2+}]_i$ oscillations indirectly support the idea of an impaired coupling.

The degree of electrical coupling is expected to be inversely related to the islet size [44]. The prevalence of poor synchronisation of $[Ca^{2+}]_i$ oscillations increased with the size of both lean and *ob*/*ob* islets. This indicates that islet hypertrophy is a causal factor in the loss of beta-cell synchrony. However, the overlap between the populations of synchronous lean islets and asynchronous *ob*/*ob* islets is such that size cannot be the only cause of desynchronisation. This conclusion is supported both by the observation of desynchronisation in 19% of clusters of 5–50 cells from *ob*/*ob* mouse islets, and by the comparison of lean and *ob*/*ob* islets matched for size. Thus, in three size-classes spanning 90% of the studied lean islets, asynchrony was much more frequent in *ob*/*ob* than lean islets. It is plausible that a functional anomaly precipitates the impact of size on beta-cell coupling. As the small synchronised islets represent a minor volume of the whole endocrine pancreas in *ob*/*ob* mice (the typical very big islets were not used deliberately), there is no doubt that poor synchronisation is a characteristic of the vast majority of the islets in these animals.

An increase in beta-cell $[Ca^{2+}]_i$ is necessary for glucose-stimulation of insulin secretion. However, it is still disputed whether the oscillations of insulin secretion are obligatorily driven by oscillations of $[Ca^{2+}]_i$ [24, 25, 28, 32] or can be induced by oscillations of metabolism modulating the efficacy of Ca^{2+} on exocytosis [13, 46]. Using normal mouse islets, we

have previously shown that the second mechanism is possible, but considerably less potent than $[Ca^{2+}]_i$ changes in shaping oscillations of secretion [47]. Our results further support the pre-eminent role of Ca^{2+}. A major observation of this study was that insulin secretion by a single islet followed the global $[Ca^{2+}]_i$ changes not only when these were regular and well synchronised in the whole organ, but also when they were irregular because of desynchronisation of the oscillations.

Glucose-induced $[Ca^{2+}]_i$ changes are perturbed in islets from the spontaneously diabetic *db*/*db* mouse [48], a model of human Type II diabetes, but the degree of synchronisation of $[Ca^{2+}]_i$ oscillations throughout the islet and the temporal correlation with insulin secretion have not been investigated. The *ob*/*ob* mouse is not a good model of Type II diabetes. Thus, its beta cells have such hyperplastic and insulin-releasing capacities that they can prevent the development of overt hyperglycaemia in the presence of the enormous insulin-resistance of peripheral tissues. Nevertheless, our findings could have important pathophysiological significance. They show that the regular pulsatility of insulin secretion by the individual islet is lost when the synchronisation of the $[Ca^{2+}]_i$ signal is altered, and thus provide a plausible mechanistic explanation for the loss of regularity of plasma insulin oscillations. We acknowledge that oscillations of insulin secretion have been reported to occur in the absence of islet $[Ca^{2+}]_i$ oscillations [13], in particular in the rat whose beta cells are steadily depolarised by glucose [49]. However, like normal mouse islets, normal human islets display regular $[Ca^{2+}]_i$ oscillations during glucose stimulation [50]. The possibility that disruption of these oscillations contributes to the perturbed pulsatility of insulin secretion in Type II diabetic patients is plausible, but will be difficult to test reliably.

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References

1. Lang DA, Matthews DR, Peto J, Turner RC (1979) Cyclic oscillations of basal plasma glucose and insulin concentrations in human beings. N Engl J Med 301:1023–1027
2. Polonsky KS, Given BD, Van Cauter E (1988) Twenty-four-hour profiles and pulsatile patterns of insulin secretion in normal and obese subjects. J Clin Invest 81:442–448
3. Porksen N, Nyholm B, Veldhuis JD, Butler PC, Schmitz O (1997) In humans at least 75% of insulin secretion arises from punctuated insulin secretory bursts. Am J Physiol 273:E908–E914
4. Lang DA, Matthews DR, Burnett MA, Turner RC (1981) Brief, irregular oscillations of basal plasma insulin and glucose concentrations in diabetic man. Diabetes 30:435–439
5. O'Rahilly S, Turner RC, Matthews DR (1988) Impaired pulsatile secretion of insulin in relatives of patients with non-insulin-dependent diabetes. N Engl J Med 318:1225–1230
6. Gumbiner B, Van Cauter E, Beltz WF et al. (1996) Abnormalities of insulin pulsatility and glucose oscillations during meals in obese non-insulin-dependent diabetic patients: effects of weight reduction. J Clin Endocrinol Metab 81:2061–2068
7. Zarkovic M, Ciric J, Stojanovic M et al. (1999) Effects of insulin sensitivity on pulsatile insulin secretion. Eur J Endocrinol 141:494–501
8. Matthews DR, Naylor BA, Jones RG, Ward GM, Turner RC (1983) Pulsatile insulin has greater hypoglycemic effect than continuous delivery. Diabetes 32:617–621
9. Paolisso G, Sgambato S, Gentile S et al. (1988) Advantageous metabolic effects of pulsatile insulin delivery in non-insulin-dependent diabetic patients. J Clin Endocrinol Metab 67:1005–1010
10. Bergsten P (2000) Pathophysiology of impaired pulsatile insulin release. Diabetes Metab Res Rev 16:179–191
11. Porksen N (2002) The in vivo regulation of pulsatile insulin secretion. Diabetologia 45:3–20
12. Gilon P, Ravier MA, Jonas JC, Henquin JC (2002) Control mechanisms of the oscillations of insulin secretion in vitro and in vivo. Diabetes 51 [Suppl 1]:S144–S151
13. Tornheim K (1997) Are metabolic oscillations responsible for normal oscillatory insulin secretion? Diabetes 46:1375–1380
14. Longo EA, Tornheim K, Deeney JT et al. (1991) Oscillations in cytosolic free Ca^{2+}, oxygen consumption, and insulin secretion in glucose-stimulated rat pancreatic islets. J Biol Chem 266:9314–9319
15. Jung SK, Kauri LM, Qian WJ, Kennedy RT (2000) Correlated oscillations in glucose consumption, oxygen consumption, and intracellular free Ca^{2+} in single islets of Langerhans. J Biol Chem 275:6642–6650
16. Ortsäter H, Liss P, Lund PE, Åkerman KEO, Bergsten P (2000) Oscillations in oxygen tension and insulin release of individual pancreatic *ob*/*ob* mouse islets. Diabetologia 43:1313–1318
17. Henquin JC, Meissner HP (1984) Significance of ionic fluxes and changes in membrane potential for stimulus-secretion coupling in pancreatic β-cells. Experientia 40:1043–1052
18. Satin LS, Smolen PD (1994) Electrical bursting in β-cells of the pancreatic islets of Langerhans. Endocrine 2:677–687
19. Valdeolmillos M, Gomis A, Sánchez-Andrés JV (1996) *In vivo* synchronous membrane potential oscillations in mouse pancreatic β-cells: lack of coordination between islets. J Physiol (Lond) 493:9–18
20. Grapengiesser E, Gylfe E, Hellman B (1988) Glucose-induced oscillations of cytoplasmic Ca^{2+} in the pancreatic β-cell. Biochem Biophys Res Commun 151:1299–1304
21. Santos RM, Rosario LM, Nadal A, Garcia-Sancho J, Soria B, Valdeomillos M (1991) Widespread synchronous $[Ca^{2+}]_i$ oscillations due to bursting electrical activity in single pancreatic islets. Pflugers Arch 418:417–422
22. Gilon P, Henquin JC (1992) Influence of membrane potential changes on cytoplasmic Ca^{2+} concentration in an electrically excitable cell, the insulin-secreting pancreatic B-cell. J Biol Chem 267:20713–20720

23. Worley JF 3rd, McIntyre MS, Spencer B et al. (1994) Endoplasmic reticulum calcium store regulates membrane potential in mouse islet β-cells. J Biol Chem 269:14359–14362
24. Gilon P, Shepherd RM, Henquin JC (1993) Oscillations of secretion driven by oscillations of cytoplasmic Ca^{2+} as evidenced in single pancreatic islets. J Biol Chem 268:22265–22268
25. Gilon P, Henquin JC (1995) Distinct effects of glucose on the synchronous oscillations of insulin release and cytoplasmic Ca^{2+} concentration measured simultaneously in single mouse islets. Endocrinology 136:5725–5730
26. Bergsten P, Grapengiesser E, Gylfe E, Tengholm A, Hellman B (1994) Synchronous oscillations of cytoplasmic Ca^{2+} and insulin release in glucose-stimulated pancreatic islets. J Biol Chem 269:8749–8753
27. Bergsten P (1995) Slow and fast oscillations of cytoplasmic Ca^{2+} in pancreatic islets correspond to pulsatile insulin release. Am J Physiol Endocrinol Metab 268:E282–E287
28. Barbosa RM, Silva AM, Tomé AR, Stamford JA, Santos RM, Rosario LM (1998) Control of pulsatile 5-HT/insulin secretion from single mouse pancreatic islets by intracellular calcium dynamics. J Physiol (Lond) 510:135–143
29. Larsson-Nyrén G, Sehlin J (1996) Comparison of the effects of perchlorate and Bay K 8644 on the dynamics of cytoplasmic Ca^{2+} concentration and insulin secretion in mouse β-cells. Biochem J 314:167–173
30. Khan A, Efendic S (1995) Evidence that increased glucose cycling in islets of diabetic *ob*/*ob* mice is a primary feature of the disease. Am J Physiol 269:E623–E626
31. Liu YJ, Tengholm A, Grapengiesser E, Hellman B, Gylfe E (1998) Origin of slow and fast oscillations of Ca^{2+} in mouse pancreatic islets. J Physiol 508:471–481
32. Jonas JC, Gilon P, Henquin JC (1998) Temporal and quantitative correlations between insulin secretion and stably elevated or oscillatory cytoplasmic Ca^{2+} in mouse pancreatic β-cells. Diabetes 47:1266–1273
33. Jonkers FC, Jonas JC, Gilon P, Henquin JC (1999) Influence of cell number on the characteristics and synchrony of Ca^{2+} oscillations in clusters of mouse pancreatic islet cells. J Physiol (Lond) 520:839–849
34. Westman S (1970) Pathogenetic aspects of the obese-hyperglycemic syndrome in mice (genotype *ob*/*ob*): I. Function of the pancreatic B-cells. Diabetologia 6:279–283
35. Oldenborg PA, Sehlin J (1997) Effects of D-glucose on chemokinesis and resting production of reactive oxygen species in neutrophil granulocytes of lean or obese-hyperglycemic mouse. Biosci Rep 17:487–498
36. Hellman B (1965) Studies in obese hyperglycemic mice. Ann NY Acad Sci 131:541–558.
37. Jonkers FC, Henquin JC (2001) Measurements of cytoplasmic Ca^{2+} in islet cell clusters show that glucose rapidly recruits β-cells and gradually increases the individual cell response. Diabetes 50:540–550
38. Gylfe E, Grapengiesser E, Hellman B (1991) Propagation of cytoplasmic Ca^{2+} oscillations in clusters of pancreatic β-cells exposed to glucose. Cell Calcium 12:229–240
39. Nadal A, Quesada I, Soria B (1999) Homologous and heterologous asynchronicity between identified α-, β- and δ-cells within intact islets of Langerhans in the mouse. J Physiol 517:85–93
40. Bertuzzi F, Davalli AM, Nano R et al. (1999) Mechanisms of coordination of Ca^{2+} signals in pancreatic islet cells. Diabetes 48:1971–1978
41. Aslanidi OV, Mornev OA, Skyggebjerg O et al. (2001) Excitation wave propagation as a possible mechanism for signal transmission in pancreatic islets of Langerhans. Biophys J 80:1195–1209
42. Gylfe E, Grapengiesser E, Liu YJ et al. (1998) Generation of glucose-dependent slow oscillations of cytoplasmic Ca^{2+} in individual pancreatic β-cells. Diabetes Metab 24:25–29
43. Ahmed M, Grapengiesser E (2001) Pancreatic β-cells from obese-hyperglycemic mice are characterized by excessive firing of cytoplasmic Ca^{2+}-transients. Endocrine 15:73–78
44. Pérez-Armendariz E, Atwater I, Bennett MVL (1995) Mechanisms for fast intercellular communication within a single islet of Langerhans. In: Huizinga JD (ed) Pacemaker activity and intercellular communication. CRC Press, London, pp 305–321
45. Rosario LM, Atwater I, Rojas E (1985) Membrane potential measurements in islets of Langerhans from *ob*/*ob* obese mice suggest an alteration in$[Ca^{2+}]_i$-activated K^+ permeability. Q J Exp Physiol 70:137–150
46. Westerlund J, Gylfe E, Bergsten P (1997) Pulsatile insulin release from pancreatic islets with nonoscillatory elevation of cytoplasmic Ca^{2+}. J Clin Invest 100:2547–2551
47. Ravier MA, Gilon P, Henquin JC (1999) Oscillations of insulin secretion can be triggered by imposed oscillations of cytoplasmic Ca^{2+} or metabolism in normal mouse islets. Diabetes 48:2374–2382
48. Roe MW, Philipson LH, Frangakis CJ et al. (1994) Defective glucose-dependent endoplasmic reticulum Ca^{2+} sequestration in diabetic mouse islets of Langerhans. J Biol Chem 269:18279–18282
49. Antunes CM, Salgado AP, Rosario LM, Santos RM (2000) Differential patterns of glucose-induced electrical activity and intracellular calcium responses in single mouse and rat pancreatic islets. Diabetes 49:2028–2038
50. Martin F, Soria B (1996) Glucose-induced $[Ca^{2+}]_i$ oscillations in single human pancreatic islets. Cell Calcium 20:409–414