

Temporal and Quantitative Correlations Between Insulin Secretion and Stably Elevated or Oscillatory Cytoplasmic Ca^{2+} in Mouse Pancreatic β -Cells

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An increase in cytoplasmic Ca^{2+} in β -cells is a key step in glucose-induced insulin secretion. However, whether changes in cytoplasmic free Ca^{2+} ($[\text{Ca}^{2+}]_i$) directly regulate secretion remains disputed. This question was addressed by investigating the temporal and quantitative relationships between $[\text{Ca}^{2+}]_i$ and insulin secretion. Both events were measured simultaneously in single mouse islets loaded with fura-PE3 and perfused with a medium containing diazoxide (to prevent any effect of glucose on the membrane potential) and either 4.8 or 30 mmol/l K^+ . Continuous depolarization with 30 mmol/l K^+ in the presence of 15 mmol/l glucose induced a sustained rise in $[\text{Ca}^{2+}]_i$ and insulin release. No oscillations of secretion were detected even after mathematical analysis of the data (pulse, spectral and sample distribution analysis). In contrast, alternating between 30 and 4.8 mmol/l K^+ (1 min/2 min or 2.5 min/5 min) triggered synchronous $[\text{Ca}^{2+}]_i$ and insulin oscillations of regular amplitude in each islet. A good correlation was found between $[\text{Ca}^{2+}]_i$ and insulin secretion, and it was independent of the presence or absence of oscillations. This quantitative correlation between $[\text{Ca}^{2+}]_i$ and insulin secretion was confirmed by experiments in which extracellular Ca^{2+} was increased or decreased (0.1–2.5 mmol/l) stepwise in the presence of 30 mmol/l K^+ . This resulted in parallel stepwise increases or decreases in $[\text{Ca}^{2+}]_i$ and insulin secretion. However, while the successive $[\text{Ca}^{2+}]_i$ levels were unaffected by glucose, each plateau of secretion was much higher in 20 than in 3 mmol/l glucose. In conclusion, in our preparation of normal mouse islets, insulin secretion oscillates only when $[\text{Ca}^{2+}]_i$ oscillates in β -cells. This close temporal relationship between insulin secretion and $[\text{Ca}^{2+}]_i$ changes attests of the regulatory role of Ca^{2+} . There also exists a quantitative relationship that is markedly influenced by the concentration of glucose. *Diabetes* 47:1266–1273, 1998

Insulin secretion is a Ca^{2+} -dependent and pulsatile process. In normal human subjects, plasma insulin concentrations oscillate at low and high frequencies (1–4). Low-frequency (ultradian) oscillations with a

period of about 120 min are usually considered to result from feedback loops linking glucose and insulin (5), although one in vitro study has recorded similar oscillations of insulin secretion from isolated rat islets perfused at constant glucose (6). High-frequency oscillations appear to have a period of about 5 min (4), which is shorter than that originally observed (1) and close to that of the oscillations of insulin secretion from the isolated and perfused pancreas (7). They may thus reflect intrinsic properties of β -cells. Both types of oscillations are disorganized in NIDDM patients and their relatives (8–10).

Changes in the concentration of cytoplasmic free Ca^{2+} ($[\text{Ca}^{2+}]_i$) are an important step in stimulus-secretion coupling in β -cells (11–13). Upon glucose stimulation, the β -cell membrane depolarizes, and oscillations of the membrane potential appear and lead to intermittent Ca^{2+} influx through voltage-dependent Ca^{2+} channels (12–15). This periodic influx of Ca^{2+} causes synchronous oscillations of $[\text{Ca}^{2+}]_i$ in all regions of the islet (16,17). Depending on the experimental conditions, both slow (2–5 min) and fast (12–20 s) oscillations of $[\text{Ca}^{2+}]_i$ have been observed in isolated mouse islets (16,17). Recently, simultaneous measurements of $[\text{Ca}^{2+}]_i$ and insulin release in the same islets have revealed a good synchrony between slow and fast oscillations of both events (18–23). Although these observations suggest that $[\text{Ca}^{2+}]_i$ changes play a regulatory role in secretion, they do not preclude the participation of other messengers. For instance, an as yet to be identified metabolic event could oscillate at the same pace and be the truly important regulator of secretion (24). Reports that oscillations of insulin secretion can occur in the absence of $[\text{Ca}^{2+}]_i$ oscillations in islet cells would support the role of metabolic oscillators (25,26). Thus, a better understanding of the links between the oscillations of β -cell $[\text{Ca}^{2+}]_i$ and insulin secretion has important pathophysiological implications.

In the present study, we measured simultaneously $[\text{Ca}^{2+}]_i$ and insulin secretion in single mouse islets. Our aim was threefold. We first investigated whether the rate of insulin secretion is stable or oscillatory when $[\text{Ca}^{2+}]_i$ in β -cells is steadily elevated. Second, we compared the efficacy of slow oscillations and of a sustained elevation of $[\text{Ca}^{2+}]_i$ in β -cells on insulin secretion. Third, we established the quantitative relationship between $[\text{Ca}^{2+}]_i$ in β -cells and insulin secretion at different glucose concentrations. The experiments were performed under conditions preventing uncontrolled variations of $[\text{Ca}^{2+}]_i$. Diazoxide, which has long been known not to affect glucose metabolism in mouse islets (27,28), was used to open ATP-sensitive K^+ channels and, thereby, prevent glucose-induced changes in membrane potential and $[\text{Ca}^{2+}]_i$ (29–31). The extracellular concentration of K^+ ($[\text{K}^+]_o$) was then

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$[\text{Ca}^{2+}]_i$, cytoplasmic free Ca^{2+} ; CV, coefficient of variation; $[\text{K}^+]_o$, extracellular concentration of K^+ ; RIA, radioimmunoassay.

raised steadily or intermittently to depolarize the membrane, stimulate Ca^{2+} entry, raise $[\text{Ca}^{2+}]_i$, and trigger insulin release. The same experimental approach has been used previously to demonstrate that glucose increases the efficacy of high $[\text{Ca}^{2+}]_i$ on insulin secretion independently from changes in the membrane potential (31–33).

RESEARCH DESIGN AND METHODS

Preparation. The pancreas from fed female NMRI mice (25–30 g) was finely chopped with scissors. After washing with a bicarbonate-buffered medium (see below), the pieces were transferred to a tube into which 2 mg collagenase (Serva, Heidelberg, Germany) per pancreas was added. After ~12 min of digestion at 37°C, during which time the tube was shaken by hand, the tissue was washed several times with cold medium supplemented with DNase (Boehringer Mannheim, Mannheim, Germany). Intact islets free of exocrine tissue were hand-picked under a stereomicroscope. They were then cultured overnight in RPMI 1640 medium (Flow Laboratories, ICN Biomedicals, Irvine, U.K.) supplemented with 10 mmol/l glucose, 10% heat-inactivated fetal calf serum, and 100 IU/ml penicillin.

Solutions. The medium used for islet isolation and for the experiments after islet culture was a bicarbonate-buffered solution containing (in mmol/l): NaCl 120, KCl 4.8, CaCl_2 2.5, MgCl_2 1.2, and NaHCO_3 24. It was gassed with O_2/CO_2 (94/6) to maintain a pH of 7.4, and it was supplemented with 1 mg/ml bovine serum albumin fraction V (Boehringer Mannheim). When the concentration of KCl was raised to 30 mmol/l (K^+ 30), that of NaCl was reduced to 94.8 mmol/l to keep the osmolarity of the medium unchanged. Ca^{2+} -free solutions were prepared by replacing CaCl_2 with MgCl_2 ; they were mixed in appropriate proportions with solutions containing 2.5 mmol/l CaCl_2 to achieve intermediate concentrations of CaCl_2 . During the experiments, the solutions with normal or high K^+ were supplemented with 250 $\mu\text{mol/l}$ diazoxide to optimize control of the membrane potential by the K^+ concentration (33).

Simultaneous measurements of $[\text{Ca}^{2+}]_i$ and insulin release. The system used to measure $[\text{Ca}^{2+}]_i$ and insulin release was similar to that previously used in the laboratory (18,20). Cultured islets were loaded with fura-PE3 for 90–120 min at 37°C in a medium containing 10 mmol/l glucose and 2 $\mu\text{mol/l}$ fura-PE3 acetoxymethyl ester (Teflabs, Austin, TX) (added from a 2 mmol/l stock solution in Me_2SO). After loading, one islet was transferred into a perfusion chamber (Intracell; Royston, Herts, U.K.) with a bottom made of a glass coverslip and mounted on the stage of an inverted microscope. The original chamber was given an oval shape, and its volume was reduced to 110 μl by an inner metal ring. The islet was held in place by gentle suction with a glass micropipette. A 19-gauge needle was placed just downstream of the islet to collect the perfusion medium as close as possible to the tissue. The islet was perfused at a flow rate of 1.8 ml/min (effluent fractions collected every 12 or 30 s) or 1 ml/min (60-s fraction collections), as indicated; each fraction therefore corresponds to 3.3 to 9.1 times the chamber volume. Continuously gassed perfusion solutions were preheated in a water bath, and a temperature controller ensured a temperature of 37.2°C ($\pm 0.3^\circ\text{C}$) close to the islet as monitored by a Thermistor (Fenwal Electronics, Framingham, MA) placed near the tissue. After switching solutions, there was a delay of 67 s before the new solution arrived in the chamber. The time between entry of the liquid into the outflow system and its collection in the tubes was 21 s. These delays have been corrected for in the figures.

The $[\text{Ca}^{2+}]_i$ was measured by dual-wavelength excitation microspectrofluorimetry. Briefly, the islet was excited successively at 340 and 380 nm, and the fluorescence emitted at 510 nm was captured by a CCD camera (Photonic Science, Tunbridge Wells, U.K.). The images were analyzed with the MagiCal System from Applied Imaging (Sunderland, U.K.), as described in detail previously (17).

Insulin assay. Insulin was measured in duplicates, either in 300- μl aliquots of the effluent fractions or in 150- μl aliquots mixed with 150 μl of medium when the effluent was collected every 12 s. The radioimmunoassay (RIA) included the following steps: addition of CaCl_2 to a final concentration of 2.5 mmol/l and addition of 1.5 mmol/l EDTA; addition of guinea pig anti-insulin serum at a final dilution of 1:1,040,000 or 1:2,080,000 (batch 619, provided by Dr. P.H. Wright, Indianapolis, IN) and incubation for 27 h at 4°C; addition of a trace amount of freshly labeled ^{125}I -insulin and incubation for 18 h at 4°C. The final total volume was 520 μl . Bound insulin was then precipitated by the addition of 2.75 ml cold absolute ethanol. Standard curves were prepared by diluting rat insulin (Novo Nordisk, Bagsvaerd, Denmark) in exactly the same medium as the samples. The qualitative sensitivity of the assay was determined for each run as previously described (34). It averaged 5.6 ± 0.4 (mean \pm SE; $n = 64$) and 2.1 ± 0.2 pg/tube ($n = 43$) for the lower and higher antibody dilutions, respectively. A 50% displacement of ^{125}I -insulin was achieved by 100 and 47 pg insulin/tube. The antibody dilution was chosen according to the expected insulin concentration in the samples. Results lower than the qualitative sensitivity of the assay (if any) were set to zero.

In each run, the intra-assay variation was estimated for low and high insulin concentrations (samples from unstimulated or depolarized islets) by use of the

relationship $\text{SD} = \sqrt{(\sum d^2/2N)}$, where d is the difference between the two results in a duplicate determination, and N is the number of duplicate determinations performed (34). The coefficient of variation (CV) was calculated as $\text{CV} = s/\text{mean}$, where $s = \text{SD}/\sqrt{2}$ (SE of each sample determined in duplicate) (35). The CV was comprised between 2.4 and 10% with a mean of ~5% for insulin concentrations measured in samples from depolarized islets, and 4.4 and 40% with a mean of 22% for the lowest insulin concentrations (just above the qualitative sensitivity of the assay) measured in samples from unstimulated islets.

Data analysis. Three methods were used to search for the possible presence of oscillations or irregular pulses of insulin secretion during sustained elevation of $[\text{Ca}^{2+}]_i$ in islets continuously depolarized with 30 mmol/l K^+ . Because false oscillations can result from experimental noise (sampling, assay), identical analyses were performed on insulin concentration profiles from three corresponding control experiments (36).

Control experiments. The medium was supplemented with rat insulin to obtain concentrations similar to those of samples obtained from depolarized islets (15–40 pg/tube). This medium was then perfused (1.8 ml/min) through the system in the absence of islet, collected every 12 or 30 s, and assayed under the same conditions as samples from real experiments. Data from these control experiments are referred to as insulin concentration profiles.

Pulse analysis with the ULTRA program. Pulse analysis has been developed to detect irregular as well as regular pulses of hormonal secretion (37). The principle of this computer algorithm is the elimination of peaks for which the increment (from nadir to peak) or the decrement (from peak to following nadir) is smaller than a certain threshold related to measurement error. The threshold for optimal detection of pulses of secretion under our experimental conditions was determined by running the ULTRA program (E. Van Cauter, University of Chicago, Chicago) on insulin concentration profiles from each control experiment and varying the threshold from 2.0 to 6.0 times the intra-assay CV until no false-positive pulse was detected. The optimal thresholds for experiments with 12- and 30-s effluent collections were 4.5 and 5.0 times the intra-assay CV, respectively. Using these thresholds, the number of false-positive pulses detected by the ULTRA program was $<1/100$ samples for both types of control experiments (good specificity), and all pulses of secretion were detected in pulsatile experiments assayed under the same condition (good sensitivity).

Spectral analysis. To avoid the influence of slow trends of secretion on spectral analysis, the best-fit curve of secretion (trend) was calculated for each experiment using a least-square regression procedure to a polynomial equation of order 1 ($y = a_0x + a_1$) to 3 ($y = a_0x^3 + a_1x^2 + a_2x + a_3$). Detrended series were then calculated by removing the trend from the original series, and spectral analysis was performed by the normalized periodogram method of Lomb, the highest detectable frequency being set at the level of the Nyquist frequency (38,39). The advantage of this method, compared with the estimation of power spectrum by way of the "fast Fourier transform" algorithm, is the possibility of testing the significance of a peak in the spectrum (the null hypothesis being that the data are independent Gaussian random values).

Sample distribution analysis. To evaluate whether samples were randomly distributed around the trend, the proportion of samples lying outside a window defined by the trend ± 2 or 3 CV was determined in each experiment and compared with the Gaussian distribution probability values.

Presentation of results. Measurements of $[\text{Ca}^{2+}]_i$ and insulin secretion from single islets are illustrated by recordings that are representative of results obtained with the indicated number of islets or are shown as means \pm SE for the indicated number of experiments. Each protocol was repeated with islets from at least four different cultures. The statistical significance between means was assessed by Student's t test. Differences were considered significant at $P < 0.05$.

RESULTS

Temporal correlation between $[\text{Ca}^{2+}]_i$ and insulin secretion rate. Insulin secretion and $[\text{Ca}^{2+}]_i$ were measured in the same single islets submitted to sustained or intermittent depolarizations. These repetitive depolarizations were applied at two frequencies, after or before the sustained depolarization, to ascertain that the technique can detect oscillations of secretion and would not miss spontaneous ones if they were present. Figure 1A and B show the results of individual representative experiments, and C and D show mean values. In one type of experiment, single islets were persistently depolarized by 30 mmol/l K^+ from the start of the perfusion. After an ~15-min equilibration period (not shown), $[\text{Ca}^{2+}]_i$ was high and stable. Slow $[\text{Ca}^{2+}]_i$ oscillations of regular amplitude, with a frequency of 0.5 or 0.2/min, were then

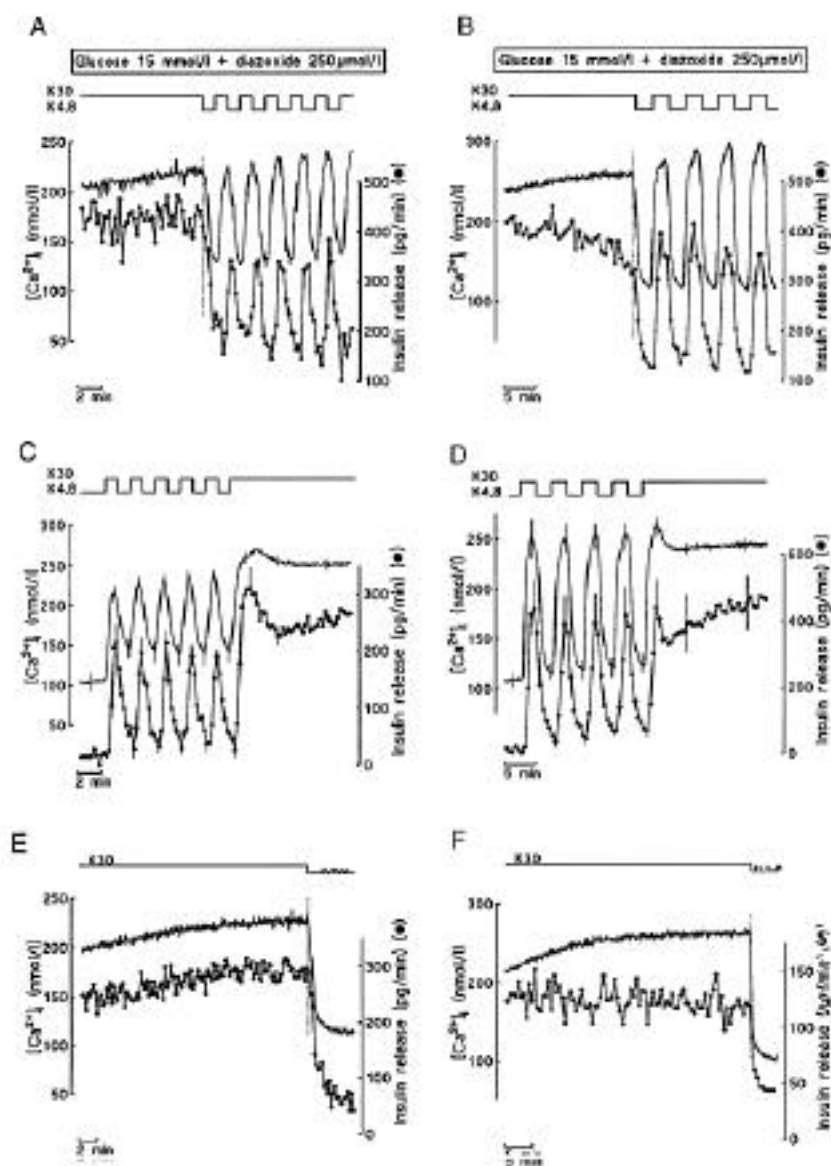


FIG. 1. Effects of sustained and intermittent depolarization on $[Ca^{2+}]_i$ and insulin release measured simultaneously in single mouse islets. After loading with fura-PE3, one single islet was perfused with a medium containing 15 mmol/l glucose and 250 μ mol/l diazoxide (flow rate 1.8 ml/min). The measurements of $[Ca^{2+}]_i$ (continuous trace) and insulin (\bullet) started after \sim 15 min of stabilization. **Protocol A:** $[K^+]_o$ was 30 mmol/l from the beginning of the perfusion. After 10 min of recording, $[K^+]_o$ was alternately reduced to 4.8 mmol/l or raised to 30 mmol/l every 1 min to induce six $[K^+]_o$ oscillations with a period of 2 min, as indicated on top of the figure. **Protocol C:** $[K^+]_o$ was 4.8 mmol/l from the beginning of the perfusion. After 2 min of recording, five $[K^+]_o$ oscillations with a period of 2 min were induced before sustained depolarization of the islet. **Protocols B and D** differ from A and C by the time scale: $[K^+]_o$ oscillations had a period of 5 min. **Protocols E and F** differ by their time scale only: $[K^+]_o$ was 30 mmol/l from the beginning of the perfusion and was lowered to 4.8 mmol/l only at the end of the experiment. The frequency of effluent sampling for insulin assay also varied with the length of the experiment. In A, C, and E (2-min time scale), insulin was measured in 12-s effluent fractions (samples diluted 1/2, anti-insulin serum diluted 1/2,080,000). In B, D, and F (5-min time scale), insulin was measured in 30-s effluent fractions (undiluted samples, anti-insulin serum diluted 1/1,040,000). A, B, E, and F: Individual experiments that are representative of results obtained with four to five islets from different cultures for each protocol. C and D: Means \pm SE for four to five different islets.

induced by alternating $[K^+]_o$ between 4.8 and 30 mmol/l every 1 min (Fig. 1A) or every 2.5 min (Fig. 1B). Whereas insulin secretion was high and did not display any apparent oscillations during continuous depolarization of the islet, regular parallel oscillations of insulin secretion and $[Ca^{2+}]_i$ were triggered by intermittent depolarizations.

In a second type of experiments, $[K^+]_o$ was kept at 4.8 mmol/l during the 15-min equilibration period (not shown) and for the first 2–3 min of measurement (Fig. 1C and D). Under these conditions, basal $[Ca^{2+}]_i$ was low and basal insulin secretion rate was barely detectable. As in the first type of experiment, intermittent depolarization triggered oscillations of $[Ca^{2+}]_i$ and insulin secretion in each individual islet. The synchrony and regularity of these oscillations are attested to by their persistence after averaging of the results (Fig. 1C and D). Subsequent continuous depolarization by 30 mmol/l K^+ induced a biphasic increase (initial peak followed by a sustained elevation) in both $[Ca^{2+}]_i$ and insulin secretion (Fig. 1C and D). When $[Ca^{2+}]_i$ was stabilized at the plateau level, no oscillations of insulin secretion were detected in any of the individual experiments (not shown). These observations

show that there exists a good temporal correlation (synchrony) between $[Ca^{2+}]_i$ and insulin secretion in single normal mouse islets. In particular, insulin oscillations follow imposed $[Ca^{2+}]_i$ oscillations at two different frequencies, but they are not detected when $[Ca^{2+}]_i$ is stably elevated.

Quantitative correlation between $[Ca^{2+}]_i$ and insulin secretion rate.

We next sought for quantitative correlations between mean $[Ca^{2+}]_i$ and insulin secretion during the four types of stimulation (sustained or intermittent) illustrated in Fig. 1A–D. Whereas mean plateau $[Ca^{2+}]_i$ was similar in all types of experiments, there was a greater variability among plateau insulin secretion rates, which we ascribe to differences in the mean size, hence, in the insulin content, of the islets. To correct for this source of variability, insulin secretion rates were expressed as a percentage of the plateau value measured in each experiment before being compared with mean $[Ca^{2+}]_i$ in the same islets. As shown in Fig. 2, there exists a good correlation between $[Ca^{2+}]_i$ and insulin release in the constant presence of 15 mmol/l glucose. This correlation was independent of the absence or presence of $[Ca^{2+}]_i$ oscillations and of the frequency of these oscillations (0.5 or

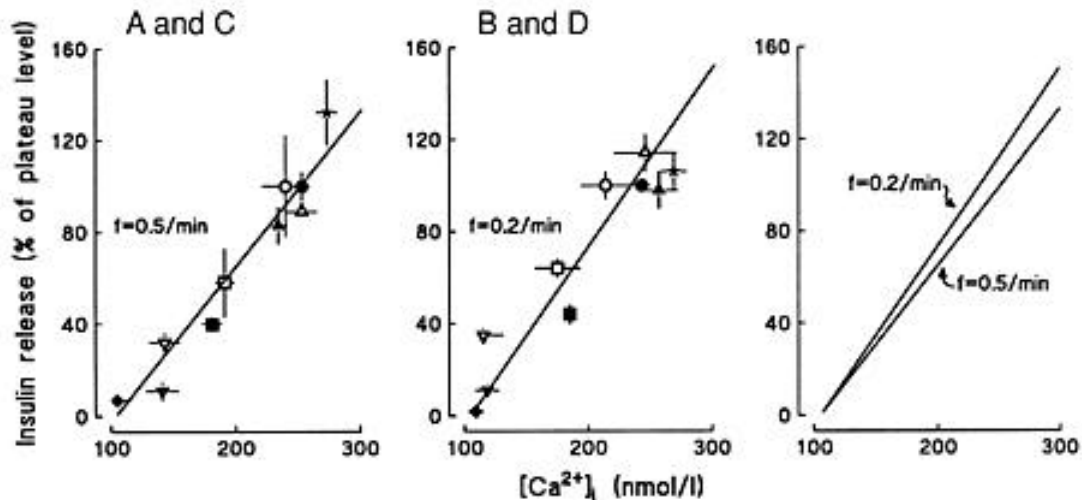


FIG. 2. Correlation between $[Ca^{2+}]_i$ and insulin release rate during sustained and intermittent depolarization of single pancreatic islets. For each experiment performed following protocols *A* through *D* illustrated in Fig. 1, mean $[Ca^{2+}]_i$ and insulin secretion rate were determined by integration over different periods indicated by open symbols (protocols *A* and *C*) or closed symbols (protocols *B* and *D*). For all protocols: \blacktriangle , peak; \blacktriangledown , nadir; \blacksquare , mean of oscillations; and \bullet , plateau during sustained depolarization (without initial 5 min in *C* and *D*). For protocols *C* and *D* only: \blacklozenge , basal before depolarization; \ast , peak of the first 5 min of sustained depolarization. Insulin secretion rates were then expressed as a percentage of the plateau values that amounted to 288 ± 63 , 374 ± 21 , 249 ± 15 , and 414 ± 80 pg/min for protocols *A*, *B*, *C*, and *D*, respectively. For all experiments at a given frequency of imposed oscillations, a linear regression curve was calculated from the mean of oscillations, basal, and plateau values. The far right panel compares the correlation lines at both frequencies. Results are means \pm SE for four to five individual experiments.

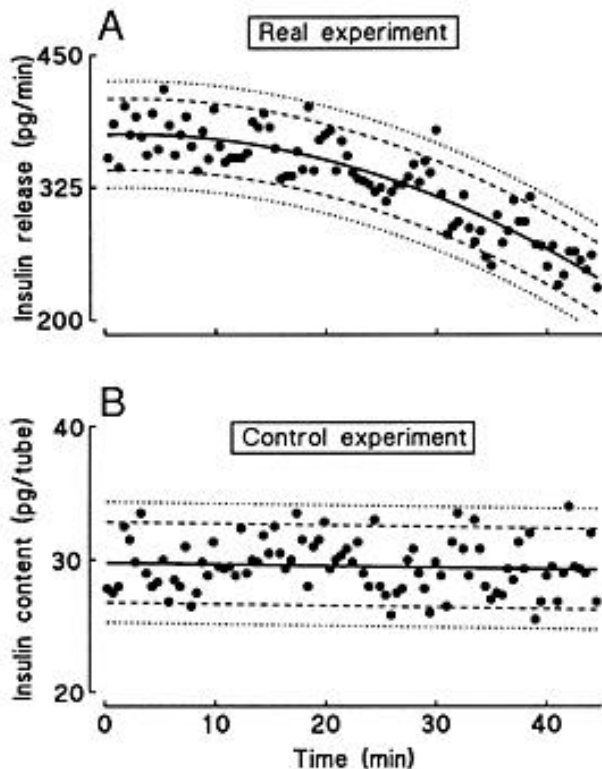


FIG. 3. Sample distribution analysis in a real and the corresponding control experiment. *A*: An experiment in which one single islet was depolarized with 30 mmol/l K^+ during 45 min (as in Fig. 1*F*). *B*: A corresponding control experiment (see METHODS for details). The insulin concentration measured in successive samples is plotted with a window corresponding to the trend (—) plus or minus two or three times the intra-assay CV (--- and lines, respectively). The proportion of samples laying outside each window was then determined and used for sample distribution analysis (see Table 1).

0.2/min) (Fig. 2, right panel). These observations suggest that imposed slow oscillations of $[Ca^{2+}]_i$ do not change the efficacy of Ca^{2+} on insulin release, at least within the duration of these short experiments.

No oscillations of insulin secretion during sustained depolarization. The experiments illustrated in Fig. 1*A–D* did not show oscillations of insulin secretion during sustained elevation of $[Ca^{2+}]_i$ in pancreatic islets. To permit mathematical analysis of the secretory data, longer experiments were performed in which one single islet was persistently depolarized with 30 mmol/l K^+ in the presence of 15 mmol/l glucose and 250 μ mol/l diazoxide, and insulin release was measured in effluent fractions collected every 12 s during 25 min or every 30 s during 45 min. Both $[Ca^{2+}]_i$ and insulin secretion rate were high and stable during these experiments, of which two examples are shown in Fig. 1*E* and *F*. The insulin secretion data obtained in each experiment were submitted to three types of analysis and compared with insulin concentration profiles from control experiments carried out as described under METHODS.

Sample distribution analysis. Figure 3 shows the results of a real experiment (protocol *F* of Fig. 1) and a corresponding control experiment, with the distribution of samples within windows of two or three CVs around the trend. The number of points outside each window was similar or lower in real experiments than in control experiments (Fig. 3 and Table 1), indicating that the variability observed in insulin release of real experiments can be accounted for by experimental noise (collection, RIA).

Spectral analysis. Spectral analysis was performed on insulin secretion data obtained after removal of the trend (see METHODS). In real experiments, no oscillations were detected in the four experiments performed with protocol *E*. In only one out of four experiments performed with protocol *F* did spectral analysis detect a frequency of 0.12/min ($P = 0.02$); this experiment is shown in Fig. 3*A*. However, a similar frequency was detected in the spectral analysis of two out

TABLE 1

Pulse characteristics determined by the ULTRA program and sample distribution analysis in insulin secretion data from depolarized single islets and in insulin concentration profiles from corresponding control experiments

Experimental protocol	Experimental data			Pulse analysis			Sample distribution analysis (% outside window)
	Insulin secretion (pg/min)	Insulin concentration (pg/tube)	Intra-assay CV (%)	Experiments with pulses (<i>n/n</i>)	Number of pulses in experiment (<i>n</i>)	Relative amplitude of pulse	
Real experiments							
<i>E</i>	245 ± 28	20 ± 2.3	6.0 ± 0.3	1/4	1	0.56	0.8 ± 0.3*
<i>F</i>	228 ± 44	38 ± 7.3	5.4 ± 0.7	1/4	1	0.59	2.0 ± 0.5
Control experiments							
<i>E</i>		15 ± 1.8	6.9 ± 0.8	2/3	1 or 2	0.44	3.7 ± 0.7
<i>F</i>		29 ± 5.5	6.4 ± 0.1	1/3	1	0.47	2.3 ± 0.7

Data are means ± SE for three to four experiments or *n*. Pulse analysis was performed with the ULTRA program on insulin secretion data from each of the four experiments performed with protocols *E* (12-s collections) and *F* (30-s collections) of Fig. 1 and on insulin concentration profiles from three control experiments mimicking both protocols (see METHODS section for details and Fig. 3 for illustration of one experiment). The relative amplitude of identified pulses was calculated by the ULTRA program as the absolute pulse amplitude (difference from nadir to peak) divided by the value at the nadir. For sample distribution analysis, the proportion of samples laying outside a window defined by the trend ±3 intra-assay CV was determined as illustrated in Fig. 3A. **P* < 0.05 vs. corresponding control experiments.

of six control experiments (0.13 and 0.09/min). When the real experiments with imposed $[Ca^{2+}]_i$ oscillations were analyzed in the same way, the corresponding oscillations of insulin secretion were consistently detected with a significance of *P* < 0.000001.

Pulse analysis. The presence of pulses of secretion was also evaluated by pulse analysis of the data as described under METHODS. The threshold was chosen to give the test both good specificity (false-positive pulses in control experiments 1/100 samples; Table 1) and good sensitivity (all pulses detected in pulsatile experiments *A–D*). In both protocols *E* and *F*, the very low number of detected pulses and the relative amplitude of these pulses were similar to those of false-positive pulses identified in control experiments (Table 1). Again, these results convincingly demonstrate that there were no significant pulses of insulin secretion in islets continuously depolarized with high K^+ in the presence of 15 mmol/l glucose and 250 μ mol/l diazoxide.

We also ascertained that the absence of oscillations of insulin secretion when $[Ca^{2+}]_i$ is steadily elevated did not result from a fortuitous choice of experimental conditions. Three other types of experiments were thus performed and analyzed as above. The islets were depolarized with 30 mmol/l K^+ in the presence of 15 mmol/l glucose but in the absence of diazoxide, or in the presence of 3 or 8 mmol/l glucose in the presence of diazoxide. Finally, islets were cultured overnight in RPMI 1640 medium containing 5 instead of 10 mmol/l glucose, a condition that favors appearance of slow $[Ca^{2+}]_i$ and insulin oscillations in glucose-stimulated islets (22,40). All results were mathematically analyzed as above. Under no conditions did we observe oscillations or pulses of insulin secretion when $[Ca^{2+}]_i$ was stably elevated by high K^+ (data not shown).

Parallel stepwise changes in $[Ca^{2+}]_i$ and insulin secretion rate. The experiments described above have shown that there exists a temporal and quantitative correlation between $[Ca^{2+}]_i$ in islet cells and insulin secretion rate in the presence of a fixed glucose concentration. However, this quantitative correlation has been established under conditions

where both $[Ca^{2+}]_i$ and insulin secretion rapidly fluctuate between high and low levels. We therefore characterized the relationship between islet $[Ca^{2+}]_i$ and insulin secretion during longer controlled stepwise changes in $[Ca^{2+}]_i$ imposed in the same islet. One single islet was continuously depolarized with 30 mmol/l K^+ in the presence of 3 or 20 mmol/l glucose and 250 μ mol/l diazoxide, and the extracellular $CaCl_2$ concentration was raised from 0.1 to 2.5 mmol/l by ~0.5-mmol/l steps (Fig. 4A). This resulted in parallel stepwise increases in $[Ca^{2+}]_i$ and insulin secretion. Remarkably, whereas $[Ca^{2+}]_i$ was not significantly affected by the glucose concentration, each step of secretion rate was larger in the presence of 20 mmol/l than in the presence of 3 mmol/l glucose. Reverse experiments were also carried out in which $CaCl_2$ was first raised to 2.5 mmol/l and then decreased stepwise. Both $[Ca^{2+}]_i$ and insulin secretion decreased in parallel steps (Fig. 4B). However, the secretion rate observed at similar $[Ca^{2+}]_i$ was always lower in the presence of 3 than of 20 mmol/l glucose, except for the first 2 min after the initial step from 0.1 to 2.5 mmol/l $CaCl_2$.

The correlations between islet $[Ca^{2+}]_i$ and insulin secretion were then determined after averaging of each parameter during the last 5 min of each step in both types of experiments (Fig. 5). Insulin secretion increased with $[Ca^{2+}]_i$ in both 3 and 20 mmol/l glucose. The relationship was fairly linear in low glucose and more sigmoidal in high glucose with, however, a linear portion between ~140 and 230 nmol/l $[Ca^{2+}]_i$. Importantly, the secretory response was much larger in high than in low glucose at all $[Ca^{2+}]_i$ exceeding ~150 nmol/l.

DISCUSSION

Changes in the concentration of glucose induce large variations in $[Ca^{2+}]_i$ in β -cells. It is, therefore, difficult to study simultaneously the respective influence of Ca^{2+} and other signals issued from glucose metabolism on insulin secretion. In previous studies, we (31,33) and others (32,41–43) have used the combination of diazoxide and high $[K^+]_o$ to clamp the membrane potential of β -cells and minimize the changes in $[Ca^{2+}]_i$ without interfering with glucose metabolism

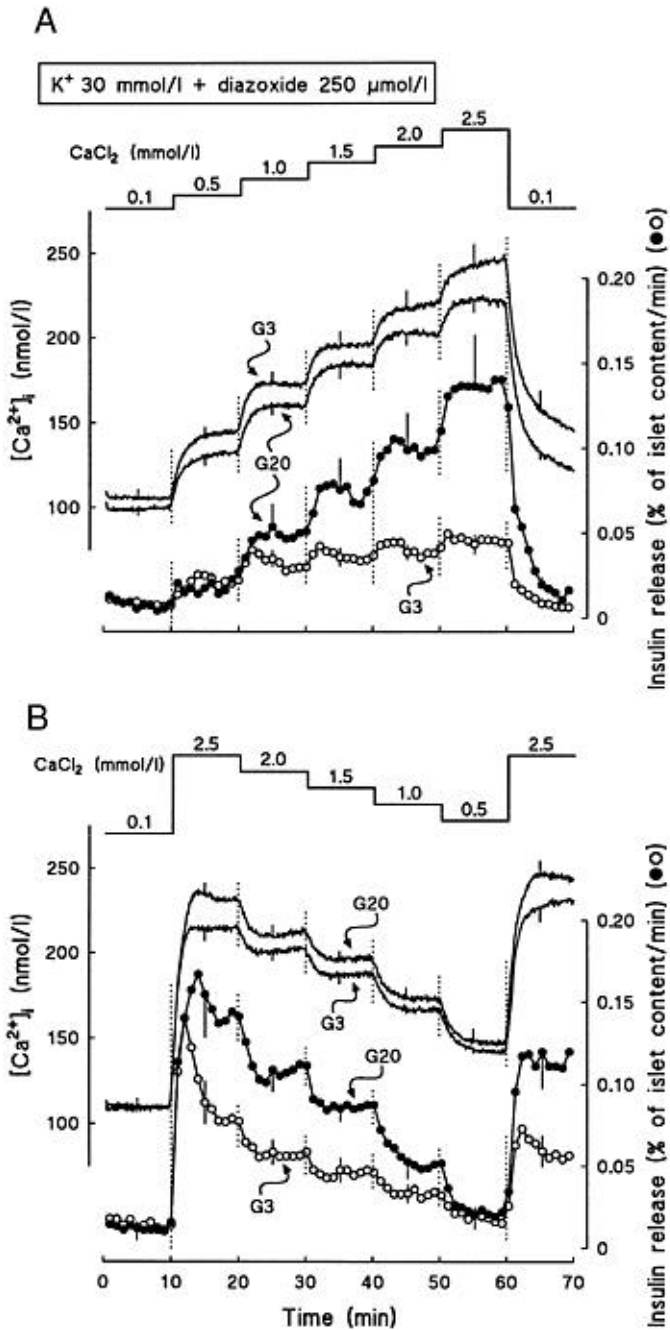


FIG. 4. Parallel stepwise changes in $[Ca^{2+}]_i$ and insulin secretion rate measured simultaneously in single islets loaded with fura-PE3. After loading, one single islet was perfused with a medium containing 30 mmol/l K⁺, 250 μmol/l diazoxide, 0.1 mmol/l CaCl₂ and 3 (○) or 20 (●) mmol/l glucose (flow rate 1 ml/min). Recordings started after 20 min of equilibration under basal conditions. The extracellular CaCl₂ concentration was then changed stepwise every 10 min, as indicated on top of each panel. Insulin, measured in duplicates in 60-s effluent collections (undiluted samples, anti-insulin serum diluted 1/2,080,000), was expressed as a percentage of the insulin content of the islet. Results are means ± SE for eight or nine islets from eight to nine different cultures. Experiments with both concentrations of glucose were performed in a paired manner on the same day with two islets from the same culture.

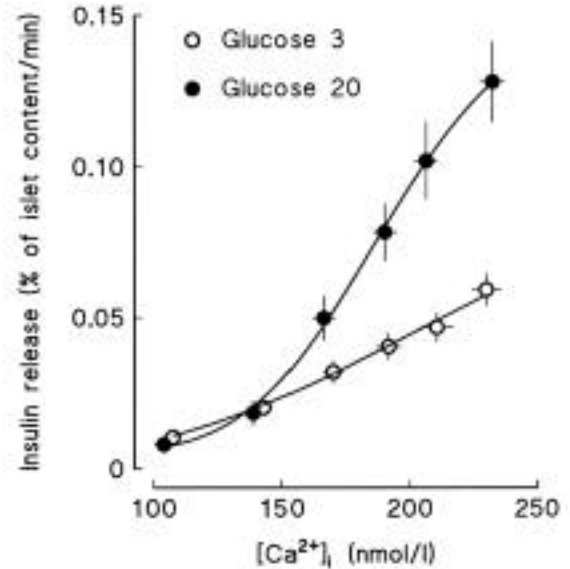


FIG. 5. Correlation between $[Ca^{2+}]_i$ and insulin secretion rate during stepwise changes in $[Ca^{2+}]_i$ in the same single islets. The results are taken from the experiments shown in Fig. 4. In each experiment, average $[Ca^{2+}]_i$ and insulin secretion rate were integrated over the last 5 min of each CaCl₂ step (over the last 3 min for the first step to 2.5 mmol/l CaCl₂ in Fig. 4B). Values are means ± SE of the results obtained at both increasing and decreasing steps to the same extracellular CaCl₂ concentration. Values were obtained in islets perfused with a medium containing 3 (○) or 20 (●) mmol/l glucose.

(27,28). This approach has permitted the characterization of a K⁺-ATP channel-independent, but Ca²⁺-dependent, mechanism of regulation of insulin secretion by glucose and other nutrients. A similar model has been used in the present study to impose oscillations or a sustained elevation of $[Ca^{2+}]_i$ in β-cells while the glucose concentration was kept constant. By measuring both parameters simultaneously in the same single islet, close temporal and quantitative correlations between $[Ca^{2+}]_i$ and insulin secretion could be established.

Temporal correlations between $[Ca^{2+}]_i$ and insulin secretion. When mouse islets are perfused with a medium containing a constant stimulatory concentration of glucose and normal $[K^+]_o$, $[Ca^{2+}]_i$ regularly oscillates in β-cells (16,17). Simultaneous measurements of $[Ca^{2+}]_i$ and insulin secretion in the same islet under these conditions have previously revealed a good synchrony between the spontaneous oscillations of both events (18–23).

In the present study, in which $[Ca^{2+}]_i$ oscillations in β-cells were imposed by cycles of depolarization and repolarization, oscillations of insulin secretion were also synchronized with those of $[Ca^{2+}]_i$, independently of their frequency (0.2–0.5/min). In contrast, insulin secretion did not oscillate when $[Ca^{2+}]_i$ was steadily elevated. The latter statement is not simply based on the examination of the secretion profiles. It is supported by three types of analysis: sample distribution analysis, spectral analysis (to detect regular oscillations), and pulse analysis with the ULTRA program (to detect unevenly distributed pulses). We also carried out control experiments (without islets), which is the only reliable way to identify artefactual frequencies introduced by technical constraints or imperfections (36). In real experiments, the number of pulses

detected by the ULTRA program was similar to that of false-positive pulses in control experiments. The frequencies of insulin oscillations detected by spectral analysis and the distribution of samples around the trend were similar in real and control experiments. It can thus be concluded that insulin secretion from normal mouse islets stimulated by glucose oscillates only when $[Ca^{2+}]_i$ oscillates in β -cells.

This conclusion conflicts with those of other recent studies. First, it has been reported that, in the presence of 3 mmol/l glucose and normal $[K^+]_o$, thus presumably when $[Ca^{2+}]_i$ is low in β -cells, basal insulin secretion slowly oscillates at a frequency of $\sim 0.4/\text{min}$ (25). These oscillations were unaffected by addition of diazoxide and persisted after 150 min of glucose deprivation, which raises questions about the nature of a possible metabolic oscillator. These observations made with giant islets from hyperglycemic *ob/ob* mice could not be verified with islets from normal mice because the rate of insulin secretion is too low in the absence of stimulation. It was also reported that insulin is released in pulses (0.3–0.5/min) of variable amplitude when $[Ca^{2+}]_i$ is stably elevated by high $[K^+]_o$ or tolbutamide (44) or by high glucose (22) in *ob/ob* mouse islets. In another study (26), batches of 50 rat islets cultured for 1–2 days were placed in a chamber and perfused with a medium containing glucose, high $[K^+]_o$, and diazoxide. Although β -cell $[Ca^{2+}]_i$ was not measured, one may also assume that it was high and stable. Yet, slow (0.17/min) oscillations of insulin secretion were observed, for which we have no explanation. The amplitude of these oscillations of secretion from single *ob/ob* mouse islets or from batches of rat islets was such that similar oscillations would have been detected without any mathematical analysis if they were present in single islets from normal mice.

It is possible that the metabolism of glucose in β -cells is characterized by oscillations in the concentration of metabolic signals serving as second messenger. Under physiological conditions, i.e., during glucose stimulation in the presence of normal $[K^+]_o$ and the absence of diazoxide, two plausible sequences might link such oscillations to those of $[Ca^{2+}]_i$. First, intrinsic oscillations of metabolism could induce the oscillations of membrane potential and $[Ca^{2+}]_i$ (24). Second, $[Ca^{2+}]_i$ oscillations could trigger the metabolic oscillations, e.g., by influencing mitochondrial dehydrogenases (45). It is thus plausible that $[Ca^{2+}]_i$ and metabolism oscillate in synchrony. Because either can independently promote exocytosis (33), it is unclear which of the two signals exerts the ultimate control on insulin oscillations. This study shows that insulin secretion does not oscillate when $[Ca^{2+}]_i$ oscillations are abrogated by sustained depolarization with high $[K^+]_o$. Whether metabolic oscillations are present under these conditions is unknown. If they are not, e.g., because they are normally driven by $[Ca^{2+}]_i$ oscillations, it is not surprising that the insulin secretion rate is stable. However, experiments using permeabilized insulin-secreting cells, in which the cytoplasmic composition is controlled, have shown that imposed oscillations of free Ca^{2+} in the medium are sufficient to trigger oscillations of secretion in the absence of any metabolic oscillation (46). If metabolic oscillations are present in our preparation of normal mouse islets during sustained depolarization with high $[K^+]_o$, e.g., because of the existence of an intrinsic oscillator, they do not seem to be able to trigger detectable oscillations of secretion in the face of stable $[Ca^{2+}]_i$. In contrast, imposed

$[Ca^{2+}]_i$ oscillations entrained synchronous insulin oscillations at two different frequencies that cannot both coincide with possible intrinsic metabolic oscillations.

We, therefore, conclude that oscillations of $[Ca^{2+}]_i$ constitute an essential minute-to-minute regulator of the oscillations of insulin secretion. In vivo, oscillations of a metabolic signal might serve to generate the oscillations of $[Ca^{2+}]_i$ (24) and/or to increase the efficacy of the synchronous oscillations of the Ca^{2+} signal on exocytosis (31–33).

Quantitative correlations between $[Ca^{2+}]_i$ and insulin secretion. The present study also established a quantitative relationship between $[Ca^{2+}]_i$ and insulin secretion in intact islets. Because of the limitations of the Ca^{2+} -dye technique, particularly when it is applied to a multicellular organ like the islet, the reported values for $[Ca^{2+}]_i$ should be regarded as mean estimates, similar to those obtained by others (19,47). Admittedly, $[Ca^{2+}]_i$ may be higher at the submembrane sites of exocytosis (48), while lower $[Ca^{2+}]_i$ may be sufficient to activate other essential processes, such as the movement of insulin granules from storage to release sites. Even if the calculated concentration is approximate, it is evident that the changes in $[Ca^{2+}]_i$ measured in whole islets adequately reflect the critical changes for secretion. Otherwise, insulin and $[Ca^{2+}]_i$ oscillations would not be synchronous. Most importantly, the relationship between $[Ca^{2+}]_i$ and insulin secretion was very different in low and high glucose. This shows that glucose increases the efficacy of Ca^{2+} on insulin secretion over the range of $[Ca^{2+}]_i$ recorded during the spontaneous oscillations induced by glucose alone under control conditions (17–22).

Mean $[Ca^{2+}]_i$ and insulin secretion in the same islets were similarly correlated when $[Ca^{2+}]_i$ was forced to oscillate (by cycles of depolarization/repolarization) or held stable at an elevated level (by sustained depolarization). The correlation was also not influenced by the frequency of the oscillations of $[Ca^{2+}]_i$. This suggests that forced slow oscillations of $[Ca^{2+}]_i$ are not more effective than a sustained elevation. In other words, slow $[Ca^{2+}]_i$ oscillations do not seem to increase the efficacy of Ca^{2+} on the insulin secretory process, at least during a relatively short period of stimulation with high $[K^+]_o$ and 15 mmol/l glucose. Similar experiments could not be performed in the presence of 3 mmol/l glucose because the rate of insulin secretion was below the detection limit of the assay in too many samples. Whether faster oscillations of $[Ca^{2+}]_i$ modulate the efficacy of Ca^{2+} is also beyond the resolution of current techniques applicable to single islets. It is also possible that $[Ca^{2+}]_i$ oscillations are important to maintain optimal β -cell function over days or longer periods of time.

In conclusion, there exists a good temporal and quantitative correlation between $[Ca^{2+}]_i$ and insulin secretion rate in normal mouse islets. Whereas the quantitative correlation is not affected by the presence and the frequency of $[Ca^{2+}]_i$ oscillations, the efficacy of $[Ca^{2+}]_i$ to stimulate insulin secretion is markedly increased by glucose. Ca^{2+} appears to play a regulatory role on secretion, the oscillations of its cytoplasmic concentration generating pulsatile insulin secretion. It may be anticipated that alterations of glucose recognition by β -cells will have two consequences: 1) a perturbation of the generation of the rhythmic triggering $[Ca^{2+}]_i$ signal and 2) an attenuation of the efficacy of Ca^{2+} on the secretory process. Both mechanisms could be involved in the pathogenesis of the abnormal pulsatility of insulin in non-insulin-dependent diabetic patients.

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