Intercellular Ca^{2+} waves sustain coordinate insulin secretion in pig islets of Langerhans

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Abstract Insulin release was investigated in parallel with changes in cytosolic calcium concentration, $[Ca^{2+}]_i$, in pig islets stimulated by glucose. After two days in culture, glucose stimulation failed to induce insulin release, and caused limited $[Ca^{2+}]_i$ changes in few cells. After ten days, insulin response was partially restored and $[Ca^{2+}]_i$ recordings revealed a slow oscillatory activity of the whole islet. Slow oscillations appeared to be due to the average $[Ca^{2+}]_i$ variations resulting from the spreading of waves throughout the islet. These waves demonstrate the reestablishment of functional cell coupling, which appears to play a critical role in insulin release.

Key words: Islets of Langerhans; Fura-2; Ca^{2+} oscillation; Ca^{2+} wave; Insulin secretion

1. Introduction

Islets behave as functional complexes where β cells operate coordinately to synchronously release insulin [1]. When dispersed, β cells in fact show higher basal insulin secretion and reduced responsiveness to glucose stimulation. The hypothesis that the islet behaves as a functional unit is reinforced by evidence that glucose-induced oscillations of $[Ca^{2+}]_i$ are synchronous in β cell clusters [2–4]. On the basis of their frequency, these oscillations have been described as either slow (< 1 min^{-1}) or fast (3–6 min^{-1}) [5–6]. Slow oscillations, that have been described to appear synchronized in adjacent cells and to occur in phase with pulsatile insulin secretion [6], are still of an unclear nature [2,3,5]. In this work, the role of intercellular communication for correct Ca^{2+} signalling, and thus for physiological insulin secretion, was investigated in pig islets maintained in culture. Digital imaging analysis of fura-2 loaded islets was used to resolve the spatiotemporal changes evoked by glucose. After ten days in culture, islets showed slow $[Ca^{2+}]_i$ oscillations that were sustained by coordinated spreading of intercellular Ca^{2+} waves.

2. Materials and methods

2.1. Islet isolation

Islets were isolated using a modification of the automated method [7] and cultured at 37°C in M199 medium, supplemented with 10% heat-inactivated swine serum, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate and 2 mM glutamine (all reagents from Biochrom KG, Seromed, Berlin, Germany). Groups of 300 hand-picked islets were placed in petri dishes. Every 48 h, the medium was changed and the number of islets was expressed as equivalent number [8]. After two or ten days in culture, 200 hand-picked islets were perifused at 37°C with an oxygenated (5% CO_2) Krebs–Ringer bicarbonate buffer (containing, in mmoles/liter: NaCl 116.0, KCl 5.0, $MgCl_2$ 2.0, $CaCl_2$ 2.5, $NaHCO_3$ 24.0 pH 7.4) supplemented with 0.5% BSA (Sigma Chemical Co., St. Louis, MO, USA). After 40 min in basal 3.3 mM glucose the islets were stimulated for 20 min with 16.7 mM glucose. Insulin secretion in the perfusate was estimated by radio immuno assay (RIA) using a commercially available kit (Incstar, Stillwater, MN, USA). In our laboratory, RIA for insulin had the following characteristics: intra-assay coefficient of variation (CV) 3%, interassay CV 5%, recovery of added doses 98–100%, minimum sensitivity 2 μU/ml. Integrated insulin release during perifusions was calculated by the standard linear trapezoidal method (Δ-AUC) and was expressed as pg/islet/20 min. The secretion index (SI) represents the ratio between the insulin secretion peak and the mean of basal values in individual experiments. Data were compared by the Students' t-test.

2.2. $[Ca^{2+}]_i$ analysis of single islets

The islets were loaded for 30 min at room temperature with 5 μM fura-2 penta-acetoxymethyl ester in Krebs-Ringer Hepes (KRH) buffer (containing, in mmoles/liter: NaCl 125.0, KCl 5.0, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.0, glucose 3.0, Hepes/NaOH 25.0 pH 7.4) supplemented with 0.0125% Pluronic F-127 (Calbiochem, San Diego Ca2+, USA). The islets were gently washed with KRH and then allowed to adhere to polyornithine-coated coverslips mounted on a thermostatted chamber (perfused by a peristaltic pump at 1 ml/min) placed on the stage of a Zeiss inverted Axiovert 135 TV microscope (Zeiss, Oberkochen, Germany). Islets were alternately excited at 340 and 380 nm by a modified Jasco CAM-230 dual wavelength microfluorimeter (Jasco, Tokio, Japan). Fluorescence images were captured by a low-light level CCD camera (Photonic Science, Robertbridge, East Sussex, UK) and fed into a digital image processor developed in the laboratory [9] where videoframes were digitized, integrated in real-time and finally stored in two hard disks. At the end of the experiment, after correction for the backgrounds, rationing and/or $[Ca^{2+}]_i$ calculation were carried out pixel by pixel on pairs of corresponding 340 and 380 nm images according to Grynkiewicz et al. [10]. In some experiment (see Fig. 3), fura-2 ratio (340/380 nm) values were first normalized based on the initial values, and then expanded in order to reveal small variations. Mean ratio values in discrete areas of interest were calculated from sequences of images to obtain quantitative temporal analysis.

3. Results

The number of isolated islets decreased during the first 48 h of culture (25.7% of the initial value, $n = 10$; $P < 0.01$, ANOVA and Scheffé test). Single cells and small fragments of islets were in fact washed off by the successive changes of medium. After this period, the number remained stable, decreasing from 25.7% at day 2, to 19.5% at day 10. A similar course was observed for islet DNA (data not shown). After 48 h in culture, the islets appeared as irregularly shaped clusters of loosely bound cells (Fig. 1A). Following ten days in culture, the islets became more compact and regularly shaped (Fig. 1B). Switching from 3.3 to

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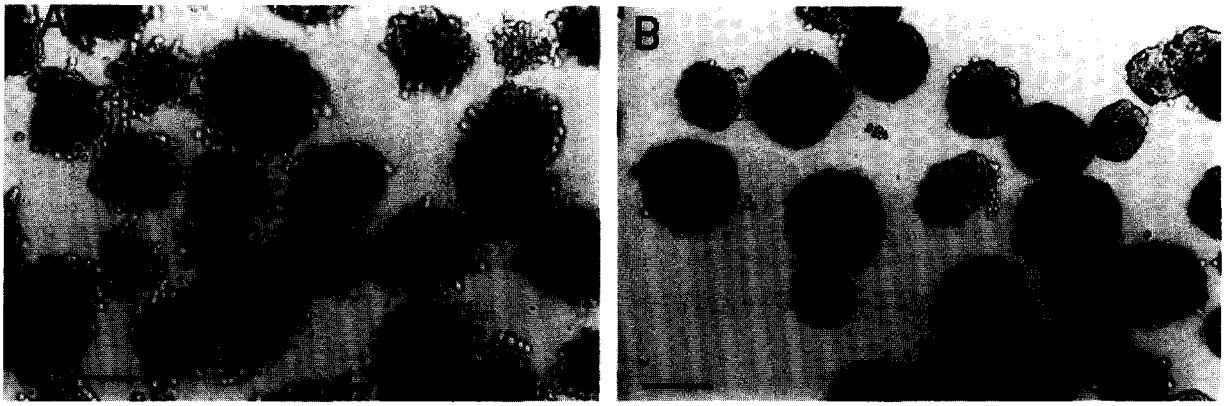

Fig. 1. Bright field images of pig islets in culture for two (A) and ten (B) days. Scale bar: 100 μm.

16.7 mM glucose, did not induce significant response in islets cultured for 48 h while in those cultured for 10 days a clear biphasic response was observed (Fig. 2A). Insulin release in response to glucose improved with culture time both in terms of variation under the curve (Δ-AUC, −5.2 ± 5.47 vs 78.09 ± 22.86 pg/islet/20 min, two vs ten day culture, respectively; $n = 17$, $P < 0.05$; Fig. 2B), and in the secretion index (i.e. the ratio between the insulin secretion peak and basal values in individual experiments; 1.61 ± 0.16 vs 2.84 ± 0.32; $n = 17$, $P < 0.05$; Fig. 2C).

The $[Ca^{2+}]_i$ responses to 16.7 mM glucose were different depending on the time in culture. Measurements from whole islets maintained in culture for two days showed negligible variations of $[Ca^{2+}]_i$. Only when the spatiotemporal aspects of the responses were analyzed in detail, small amplitude oscillations of $[Ca^{2+}]_i$ could occasionally be appreciated, sustained by one or a few identifiable cells within the islet, the others being completely unresponsive (Fig. 3). After ten days in culture, the responsiveness of the whole islets to glucose stimulation was restored. Increasing the concentration of glucose from 3.3 to 16.7 mM resulted in a widespread elevation of the $[Ca^{2+}]_i$ basal levels often associated with a slow oscillatory activity (~1 min^{-1}; Fig. 4A). Addition of excess EGTA to the medium (calculated $[Ca^{2+}] < 10^{-8}$ M) rapidly blocked these cyclic variations (data not shown). During the oscillatory activity, the videoimaging analyses of the islets revealed a periodic initiation of $[Ca^{2+}]_i$ rise from discrete areas, and their progressive spreading as waves into the neighbouring regions. Unfortunately, the spatiotemporal aspects of the signal propagation were clearly recognizable only for brief periods since waves, by spanning multiple optical focal planes within the islets, often ran out of focus. Fig. 4B shows (top left) the initial $[Ca^{2+}]_i$ level in the presence of 3.3 mM glucose followed by a gallery of images taken at 3 s intervals that represents the spatiotemporal evolution of a single slow $[Ca^{2+}]_i$ oscillation (marked by the red bar in Fig. 4A) after glucose stimulation. A $[Ca^{2+}]_i$ transient starts from an elevated interspike level and gives rise to an intercellular wave that propagates clockwise, with constant speed (~10 μm/s), along the islet equator. It should be noticed that two cells which had shown no variations in the basal $[Ca^{2+}]_i$ levels after switching to 16.7 mM glucose, were also unaffected by the passing wave. The temporal analyses (Fig. 4C) from the whole islet (trace w) and from individual cells (1–7) successively invaded by this wave highlight similarities in the response pattern and further confirm the delayed, sequential activation of the cells (see arrowheads). From the comparative analysis of the traces it is evident that the slow oscillation measured in the whole islet results from the sum of the $[Ca^{2+}]_i$ transients fired by the passing wave. The calcium responsiveness to glucose appeared, therefore, significantly improved in the islets maintained in culture for longer time. In fact, while coordinate oscillations were ob-

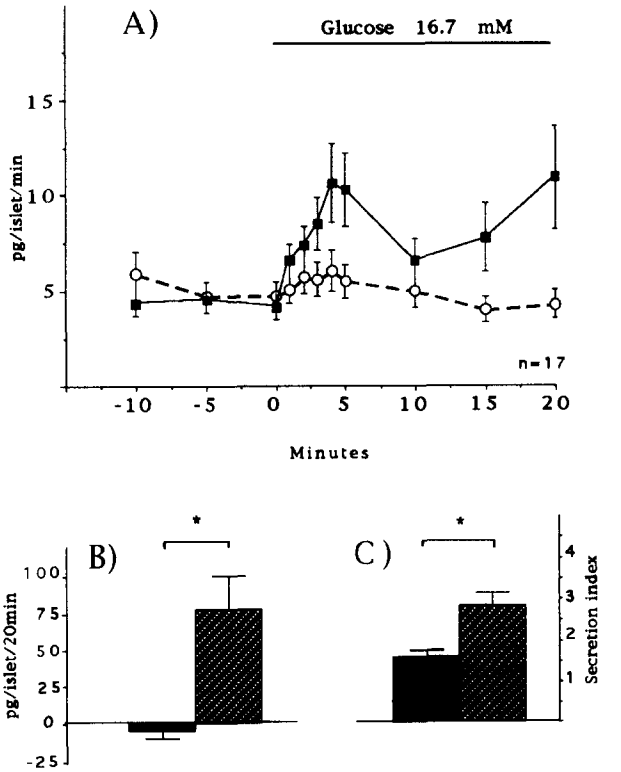

Fig. 2. Panel A shows insulin release (expressed as pg/islet/min) from 200 islets in response to 16.7 mM glucose (time 0). The kinetics were different after two (open circles) or ten (filled squares) days in culture. In B and C the total insulin secretion is expressed as the variation of the area under the curve (Δ-AUC, in pig/islet/20 min) or as the secretion index. Black and dashed columns represent data from islets cultured for two or ten days, respectively. Values are expressed as the mean ± S.E. (*$P < 0.05$, $n = 17$).

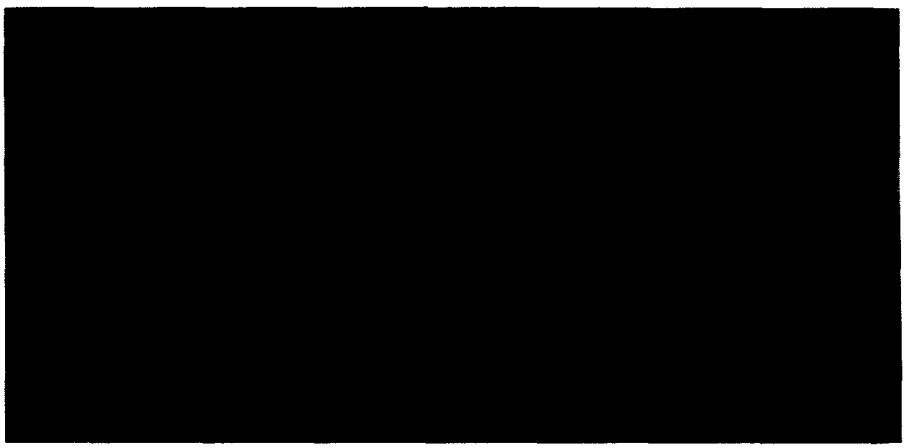

Fig. 3. Pseudocolor representation of $[Ca^{2+}]_i$ variation induced by 16.7 mM glucose in an islet maintained in culture for two days. Images were collected 3 s apart (top left to bottom right). Few cells show fluctuations in $[Ca^{2+}]_i$ without coordination. In this figure fura-2 ratio (340/380 nm) values were first normalized based on the initial values (3.3 mM glucose) and represented according to the calibration bar to the right where lowest values are coded blue.

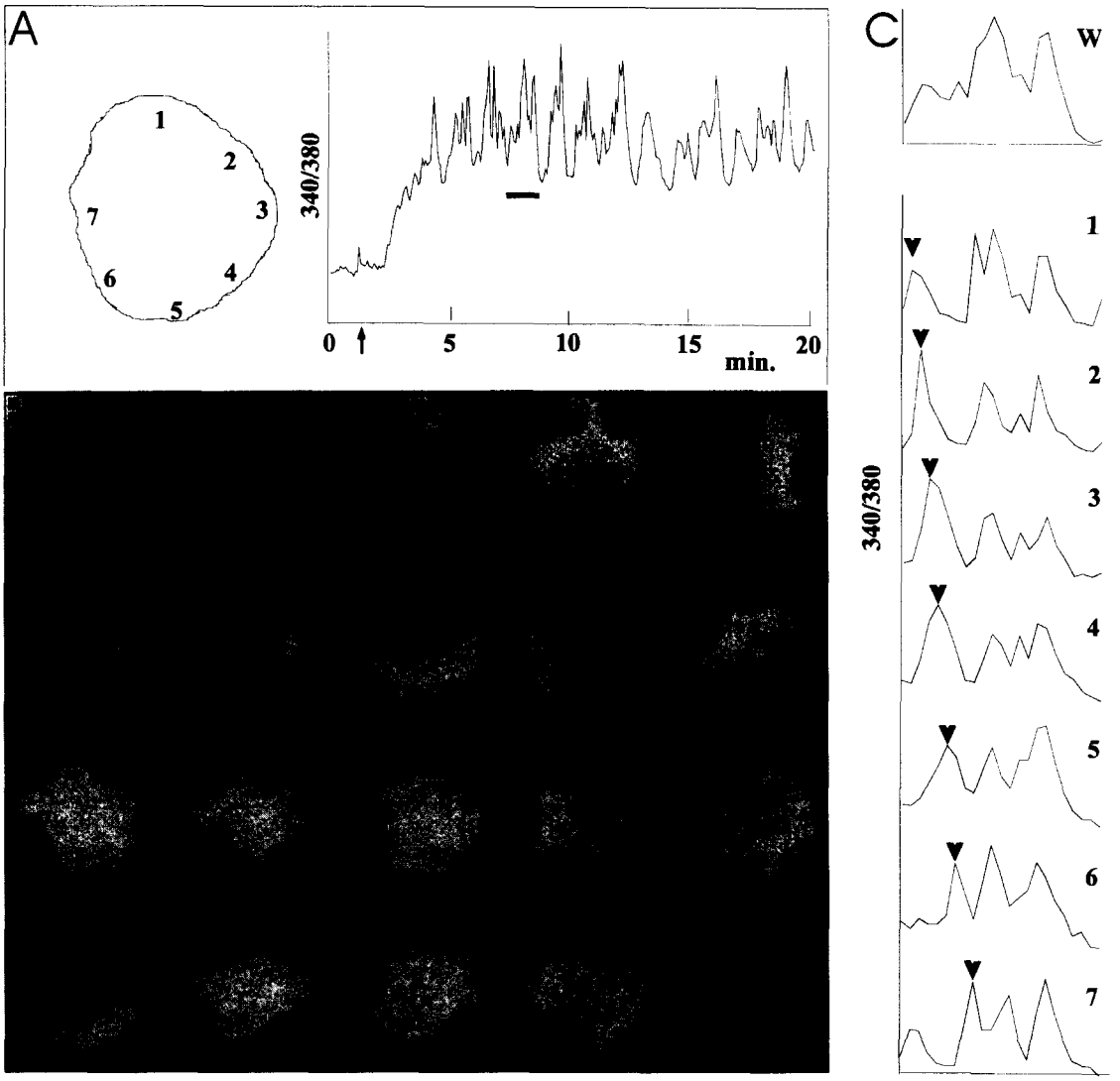

Fig. 4. (A) Temporal plot of the whole islet $[Ca^{2+}]_i$ changes after glucose stimulation (arrows). The numbers on the drawing to the left identify the positions of the individual cells that were analyzed. The spatiotemporal aspects of the slow frequency oscillation marked by the red bar (1 min) in A are reported in the gallery of images in B and in the traces in C. The top left image in B shows the $[Ca^{2+}]_i$ in presence of 3.3 mM glucose while the following images illustrate the wave starting in the cell in position 1 (top-left to bottom right; images 3 s apart). In C, the same $[Ca^{2+}]_i$ oscillation is shown in the whole islet (w) and in the cells identified in the drawing in A (1–7). Arrowheads highlight the delayed, sequential activation of the different cells.

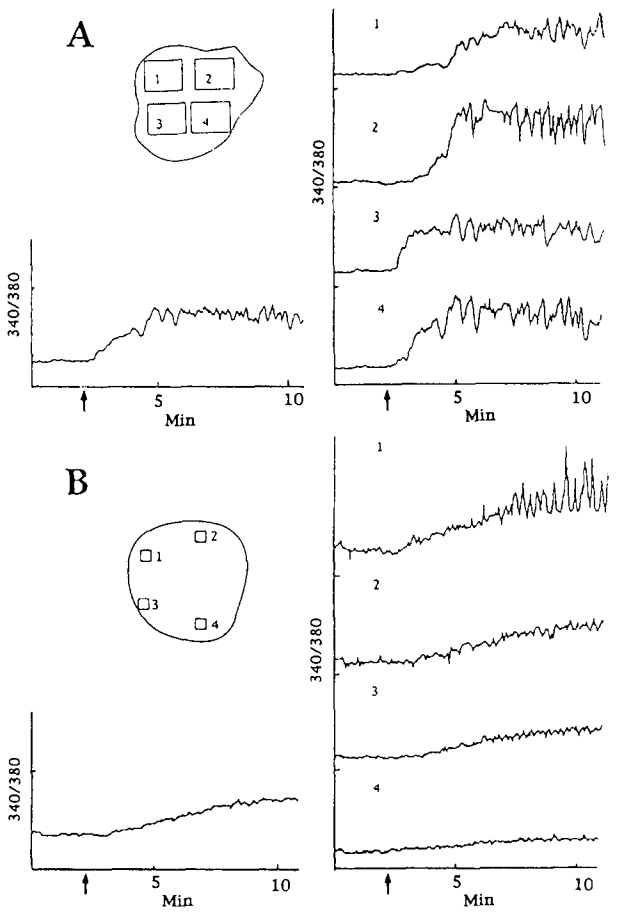

Fig. 5. $[Ca^{2+}]_i$ changes after glucose stimulation (arrows) in two islets maintained in culture for ten days. Temporal plots on the left are from the whole islet, while those on the right refer to groups of cells (A) or single cells (B) identified in the corresponding drawings.

served only in a single islet after two days, (1/8), they were present in the majority of islets after ten days (9/12; $P < 0.05$, Fischer exact test), either with a pattern common to the whole islet (Fig. 4A; 3/12) or with distinct patterns in different multicellular regions (Fig. 5A; 6/12). Within the remaining islets the responses to glucose were restricted to single cells only and highly variable (Fig. 5B; 3/12).

4. Discussion

Pig islets of Langerhans represent a multicellular system which is considered a possible attractive alternative for transplantation in humans [11]. However, in vitro studies showed a deficit of insulin secretion from pig islets with respect to those obtained from human and rat [12]. Our results clearly indicate that the isolation procedure causes tissue loss revealed by the decrease of islet number during the first two days in culture. Under these conditions, in agreement with previous studies, islets were largely unresponsive in terms not only of insulin secretion [8], but also of $[Ca^{2+}]_i$ changes. After ten days in culture the general appearance of the islets was greatly improved in agreement with previous observations [8]. The reestablishment of contacts within islet cells was found to be crucial to restore not only insulin release [13] but also $[Ca^{2+}]_i$ oscillatory activity in response to glucose stimulation. In this study we focused our attention on the mechanisms that sustain this slow frequency oscillatory activity since it is considered to be at the basis of the pulsatile secretion of insulin [6], thus playing a crucial role in the control of glycaemia both in physiological and pathological conditions [14–16].

Previous studies revealed that elevation of extracellular glucose concentrations increases not the frequency, but rather the amplitude of both slow oscillations and pulsatile insulin secretion, thus suggesting the recruitment to secretion of previously silent cells [17]. Although fura-2 imaging results showed evidence of propagation of $[Ca^{2+}]_i$ oscillations in small β cell clusters [2], no conclusive indications on the mechanism sustaining the progressive entrainment of cells into the dominant oscillatory pattern are available. Our results show that slow oscillations are not strictly synchronous in the different regions of pig islets but represent the spatiotemporal integration of Ca^{2+} waves progressively recruiting cells within the islet. Therefore, the asynchronous spatiotemporal aspects of $[Ca^{2+}]_i$ signalling appear consistent with the view that oscillations in pig islets are not the result of a collective electrical coupling of the cells (the channel sharing hypothesis; [18]) but rather governed by metabolic coupling via small messengers, such as Ca^{2+}, cAMP or inositol 1,4,5-trisphosphate [19]. Their diffusion through gap junctions could, then, dynamically control the excitability of the cells and thus different steps of the insulin secretion process.

In conclusion, our results on pig islets of Langerhans provide new insights in Ca^{2+} signalling mechanisms that are basic to both pulsatile insulin release and recruitment to secretion of cells, two processes which are crucial in the function of the endocrine pancreas and have important implications in the pathophysiology of diabetes.

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