Mechanisms of Coordination of Ca^{2+} Signals in Pancreatic Islet Cells

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Within pancreatic islet cells, rhythmic changes in the cytosolic Ca^{2+} concentration have been reported to occur in response to stimulatory glucose concentrations and to be synchronous with pulsatile release of insulin. We explored the possible mechanisms responsible for Ca^{2+} signal propagation within islet cells, with particular regard to gap junction communication, the pathway widely credited with being responsible for coordination of the secretory activity. Using fura-2 imaging, we found that multiple mechanisms control Ca^{2+} signaling in pancreatic islet cells. Gap junction blockade by 18 α-glycyrrhetinic acid greatly restricted the propagation of Ca^{2+} waves induced by mechanical stimulation of cells but affected neither Ca^{2+} signals nor insulin secretion elicited by glucose elevation. The source of Ca^{2+} elevation was also different under the two experimental conditions, the first being sustained by release from inner stores and the second by nifedipine-sensitive Ca^{2+} influx. Furthermore, glucose-induced Ca^{2+} waves were able to propagate across cell-free clefts, indicating that diffusible factors can control Ca^{2+} signal coordination. Our results provide evidence that multiple mechanisms of Ca^{2+} signaling operate in β-cells and that gap junctions are not required for intercellular Ca^{2+} wave propagation or insulin secretion in response to glucose. *Diabetes* 48:1971–1978, 1999

Glucose-induced insulin release is a complex mechanism designed to meet the metabolic requirements of the organism while preventing the downregulation of the peripheral receptors for the hormone and the desensitization of β-cell secretory machinery (1). The pulsatile pattern of insulin secretion, which has been observed both in vivo and in vitro, could very well satisfy these requirements. Indeed, loss of cyclic variations of the hormone in plasma has been proposed to play a pathogenetic role in the progression to overt diabetes (2). To date, little is known about the mechanisms that govern this specific pattern of insulin delivery, although the functional coupling among both homologous and heterologous pancreatic islet cells is considered to be a necessary prerequisite to guarantee coordinate and proper secretion of insulin in conditions of physiological stimulation (3). Accordingly, when islet architecture is altered, a concomitant impairment of insulin secretion is observed, which recovers as soon as contacts among islet cells are re-established (4). One line of evidence has led to the proposal that the coordination of β-cell secretory function is mainly regulated by direct cell-to-cell communication mediated by gap junctions (5). Such a conclusion is based on the evidence of electrical (6) and metabolic (7) coupling and on the observation that gap junctions increase in number during insulin stimulation (8) and that conditions promoting gap junction formation also improve insulin production (9). According to this hypothesis, gap junction opening could either confer electrical coupling to cells, or promote cell-to-cell transfer of small second messengers, such as Ca^{2+} and inositol 1,4,5-trisphosphate (IP_3), thus favoring the propagation of intercellular Ca^{2+} waves (10). The potential role of gap junctions in the functional coupling of pancreatic islet cells was recently emphasized by studies showing that, in response to glucose stimulation, the cytosolic concentration of calcium ($[Ca^{2+}]_i$) oscillates in synchrony with insulin secretion (11,12). This hypothesis is, however, in contrast to the evidence that electrical and metabolic coupling in β-cells is not extended to the whole islet, but is rather restricted to only few cells (13,14). In addition, the slow $[Ca^{2+}]_i$ oscillations, which had previously been described to be synchronous within individual islets (11,12,15), often revealed, on closer analysis, a sequential evolution in different regions and were, therefore, described as the spatiotemporal integration of Ca^{2+} wave spreading (16). Similar evidence for complex signal propagation was provided by studies on the whole-islet electric activity (17). These results, in their entirety, are compatible with a functional coordination of insulin secreting cells taking place via the dynamic control of gap junctional coupling (9). Alternatively, paracrine factors might coordinate β-cell activity (3,18–22), as previously reported in other cell types (23–26), including insulin-secreting cells (27).

In the current study, we investigated the mechanisms that sustain Ca^{2+} wave propagation in both isolated pancreatic islets and pancreatic islet cell monolayers. We report that proper re-aggregation of previously dispersed cells is able to completely restore gap junctional coupling, as well as glucose responsiveness, both in terms of rhythmic propagation of

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AGA, 18 α-glycyrrhetinic acid; BSA, bovine serum albumin; $[Ca^{2+}]_i$, cytosolic concentration of calcium; HELSS, haloenol lactone suicide substrate; KRH, Krebs-Ringer solution buffered with HEPES; Ry, ryanodine; SERCA, sarco/endoplasmic reticulum ATPase; Tg, thapsigargin.

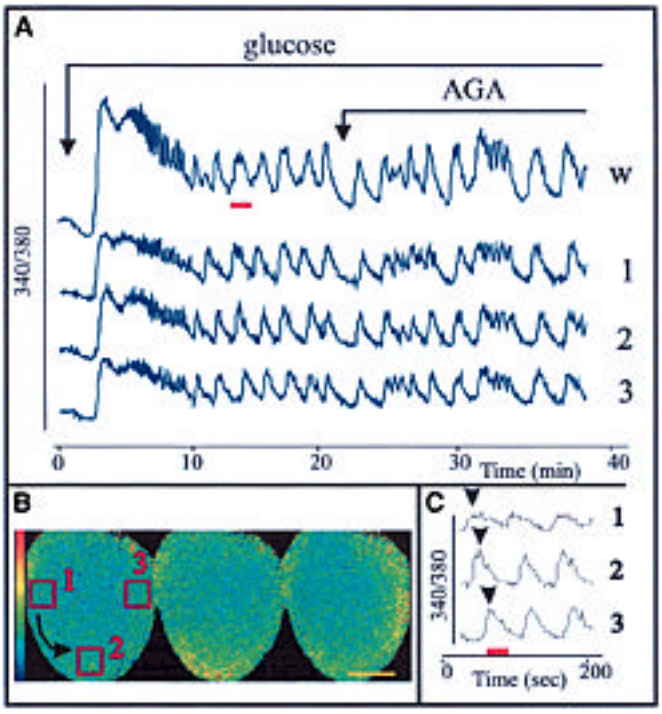

FIG. 1. $[Ca^{2+}]_i$ changes upon glucose stimulation in an islet maintained for 3 days in culture. The $[Ca^{2+}]_i$ changes after administration of 10 mmol/l glucose are illustrated as temporal plots (*A*) and as pseudocolor images (*B*). The traces in *A* were recorded from the whole islet (w) and from the three peripheral regions outlined in *B*. The series of images (from left to right, 3 s apart) shows the spatiotemporal aspects of the slow-frequency oscillation marked by the red line in *A* and *C*. An intercellular Ca^{2+} wave propagates alongside the periphery of the islet moving counterclockwise (arrow in the first image). The arrowheads in *C* emphasize the sequential activation in the different regions. Exposure to 20 µmol/l AGA did not cause blockade of the oscillatory activity (20 of 20 islets). Bar = 50.0 µm.

Ca^{2+} waves among the cells and of insulin secretion. We also show that distinct forms of Ca^{2+} signal propagation coexist in β-cells. Contrary to expectations, neither Ca^{2+} signaling nor hormone secretion induced on glucose stimulation was influenced by gap junctional coupling; rather, both parameters appeared to be controlled by the release of soluble component(s) able to modulate glucose-mediated activity.

RESEARCH DESIGN AND METHODS

Pancreatic islet isolation. The islets were isolated from CD-1 mice (25–30 g body wt) by collagenase (type V, 1 mg/ml) (Sigma Chemical, St. Louis, MO) digestion and purified by density gradient (Histopaque-1077; Sigma). Islets were then cultured at 37°C in a humidified atmosphere with 5% CO_2 in M199 medium, supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.0 mmol/l glutamine (Seromed Biochrom, Berlin).

Pancreatic islet cell monolayers. Pancreatic islets were dispersed as single cells by a brief digestion (22°C for 2 min) in Non-Enzymatic Cell Dissociation Solution (Sigma) supplemented with 1 mg/ml trypsin and 0.25 mg/ml DNAse. The cells were finally transferred onto glass coverslips pretreated with poly-L-ornithine (50 µg/ml). After 4 days of culture, cells started to form monolayers that were maintained in culture for up to 15 days under standard conditions. Immunohistochemical staining for insulin (Biogenesis, Poole, England, U.K.) and glucagon (Sigma) was performed to characterize the cell phenotypes. Number of preparations refers to the number of isolations from different animals.

Insulin secretion in pancreatic islet cell monolayers. After 6 days of culture, the insulin released from monolayers in response to glucose was assessed by either static incubation or constant perifusion (150 µl/min) with a Krebs-Ringer solution buffered with HEPES (KRH; containing, in millimoles per liter: NaCl, 125.0; KCl, 5.0; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.0; HEPES/NaOH, 25.0, pH 7.4) plus 0.1% bovine serum albumin (BSA). After 20 min of equilibration with 3.3 mmol/l glucose, islet cell monolayers were stimulated with 10 mmol/l glucose. Under perifusion, samples (3-min perifusate) were collected before, at the onset of, and 5 and 10 min after administration of the stimulus. All samples were frozen at –20°C, and insulin was assessed by radioimmunoassay, using a commercial kit (Incstar, Stillwater, Minnesota, MN). Data are expressed as means ± SD.

$[Ca^{2+}]_i$ analysis. The digital fluorescence-imaging microscopy system was built around a Zeiss inverted Axiovert 135 TV microscope (Zeiss, Oberkochen, Germany). Excitation light was from a modified Jasco CAM-230 dual wavelength microfluorimeter (Jasco, Tokyo). Fluorescence images were collected at a middle plane of the cells/islets by an intensified CCD camera (Photonic Science, Robertbridge, East Sussex, U.K.) and fed into a digital image processor where video frames were digitized and integrated (typically 10 consecutive frames) in real time. The images were then processed to convert fluorescence data to $[Ca^{2+}]_i$ images (340/380 nm excitation wave-length ratio method). The sampling of ratio images ranged from 0.3 Hz (standard) to 2 Hz.

At the beginning of each experiment, islets and monolayers were incubated at 22°C with fura-2 penta-acetoxymethylester (2–5 µmol/l, dissolved in KRH supplemented with 0.0125% Pluronic F-127 [Calbiochem, San Diego CA]) for 25 min. At the end of this period, they were gently rinsed in KRH and examined by fura-2 videomicroscopy within a specially designed microchamber (<10 µl volume) kept at 37°C under continuous perfusion (150 µl/min, average speed of visible debris in the flux ~100 µm/s). Apyrase; 18α glycyrrhetinic acid; glycyrrhizic acid; thapsigargin; ryanodine; L-N-(iminoethyl)-ornithine; suramin; free fatty acid BSA; nifedipine; exendine 9–39; and wortmannin were from Sigma. rp-cAMP, haloenol lactone suicide substrate (HELSS), and RO-8122 were from Calbiochem.

Cross-correlation analysis. The possible correlation among the responses of cells belonging to physically separated monolayers was investigated by means of Fourier analysis and cross-correlation computation. Transfer functions were computed according to standard procedures (28) and analyzed according to standard system identification approaches (29), using MATLAB software environment (The Math Works, Natick, MA). Cross-correlations were computed on successive overlapping sections of the $[Ca^{2+}]_i$ measurement recordings (128 samples per record, corresponding to 384 s - 24 s apart), and normalized to the square root of the variance product, so as to yield values ranging from 0 (no correlation) to 1.0 (perfect cross-correlation). The value and location of the cross-correlation maximum (for time-lag values between 0 and 60 s) were respectively recorded as indicators of the degree of correlation and time delay between the responses.

Dye coupling studies. Individual cells of the monolayers were microinjected (Compic automatic micro-injection system; Cellbiology Trading, Hamburg, Germany) with a Ca^{2+}/glucose-free KRH solution containing 4% Lucifer yellow (Sigma). Fluorescence images of the dye distribution were acquired from monolayers either maintained in KRH or exposed to glycyrrhetinic acid.

RESULTS

Glucose-induced Ca^{2+} signaling in isolated pancreatic islets. In a preliminary series of experiments, Langerhans islets, isolated from mice, were analyzed by fura-2 videomicroscopy to study spatiotemporal $[Ca^{2+}]_i$ changes in response to glucose stimulation. An increase in glucose concentration from 3.3 to 10 mmol/l promoted the generation of typical slow $[Ca^{2+}]_i$ oscillations (mean frequency 0.8 ± 0.4/min, ranging from 0.2 to 1.4/min, n = 25) that, at times, were observed to travel as Ca^{2+} waves throughout the islet (Fig. 1), similar to what has been described in pig islets (16). To investigate the nature of the functional coupling responsible for the synchronization of glucose-induced Ca^{2+} signals, isolated pancreatic islets were exposed (up to 40 min) to 1–20 µmol/l 18 α-glycyrrhetinic acid (AGA), a specific inhibitor of gap junctions (26,30–31). Exposure to this agent did not affect the slow $[Ca^{2+}]_i$ oscillatory activity developed in response to glucose (20 of 20 islets) (Fig. 1), thus suggesting that direct coupling among cells is not required under these conditions. Unfortunately, despite the remarkable potential interest in these results, it was problematic to further study these issues in the isolated pancreatic islets. On the one hand, it was difficult to study the spatiotemporal aspects of Ca^{2+} signals as they often ran out of focus, by spanning multiple optical focal planes within the islets; and, on the other hand, it was of concern the

extent to which drugs could access the inner core of this complex micro-organ sheathed with a tight capsule.

Functional coupling properties in pancreatic islet cell monolayers. We set up the conditions for studying monolayers of functional pancreatic islet cells (see METHODS), a type of preparation in which drug diffusion is rapid and proper analysis of Ca^{2+} signals can be performed. Re-aggregation of both α- and β-cells (~10% and ~85%, respectively, as evaluated by immunostaining for glucagon and insulin) took place in a few days, and, after one week, monolayers were studied.

Gap junctional coupling among cells was assessed by two independent approaches. First, individual cells were microinjected with Lucifer yellow, and dye labeling was recorded soon thereafter. Under control conditions, the fluorescence remained confined to a single cell in a small percentage of cases (26%), whereas, in general, two (58%), three (10%), or four (6%) cells were labeled (n = 63 from 7 preparations). Second, dye transfer was virtually abolished when preparations were preincubated for 20 min with 20 µmol/l AGA, so that Lucifer yellow appeared confined to the microinjected cell in the majority of cases (81%), with a maximum recorded signal of two cells in the remaining cases (19%, n = 58 from 5 preparations). A different strategy was adopted to study direct cell-to-cell coupling under stimulatory conditions. Monolayers were conventionally loaded by fura-2AM and then mechanically stimulated in absence of perifusion by gently applying the fire-polished tip of a glass microelectrode onto a cell. A single Ca^{2+} wave was observed to spread (average speed ~50 µm/sec) from the excited cell to the surrounding cells (~100) both in the presence and in the absence of extracellular Ca^{2+} (n = 5 from 5 preparations) (Fig. 2*A*). The wave was prevented by 20-min pretreatment with 1 µmol/l thapsigargin (Tg) (n = 4 from 4 preparations), an irreversible blocker of the sarco/endoplasmic reticulum ATPases (SERCAs), which pump Ca^{2+} into the intracellular stores, thus suggesting a direct involvement of cytoplasmic Ca^{2+} pools. The extent of Ca^{2+} wave propagation was considerably reduced after preincubation (10 min) with either 100 µmol/l suramin (a blocker of purinergic receptors, n = 4 from 4 preparations) (Fig. 2*B*) or 20 µmol/l AGA (n = 4 from 4 preparations) (Fig. 2*C*), and remained virtually confined to the mechanically stimulated cell(s) after combined treatment with the two drugs (n = 5 from 5 preparations) (Fig. 2*D*). These results confirmed that functional coupling is reestablished in pancreatic islet cell monolayers after one week, and that this type of preparation is a valuable model to study the physiology of pancreatic islets.

Glucose responsiveness in pancreatic islet cell monolayers. Exposure to 10 mmol/l glucose evoked slow $[Ca^{2+}]_i$ oscillations (Fig. 3) in a high percentage of preparations (45 of 70 monolayers) with a frequency that was comparable to that recorded in isolated islets (0.6 ± 0.3/min, ranging from 0.2–1.4/min, n = 45). The videoimaging analysis highlighted the

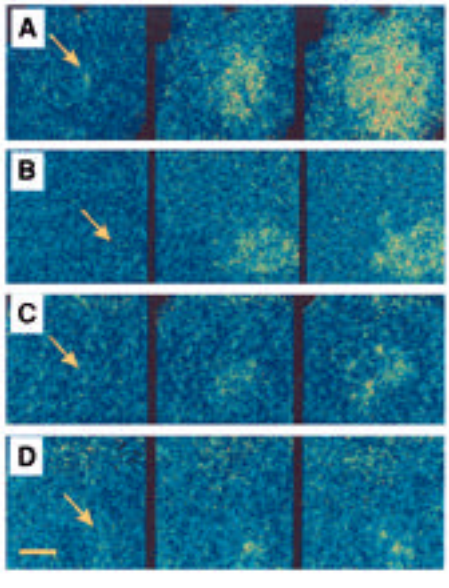

FIG. 2. $[Ca^{2+}]_i$ changes upon mechanical stimulation in pancreatic islet cell monolayers. The series of pseudocolor images (1 s apart) illustrate the $[Ca^{2+}]_i$ changes induced by gently applying a fire-polished micropipette onto a monolayer (arrows) maintained in Ca^{2+}-free medium. *A* shows a $[Ca^{2+}]_i$ wave starting from the stimulated cell and invading the neighboring ones (representative of 5 experiments from 5 preparations). The propagation was considerably reduced after 10 min preincubation with either 100 µmol/l suramin (*B*, representative of 4 experiments from 4 preparations) or 20 µmol/l AGA (*C*, representative of 4 experiments from 4 preparations), and was virtually confined to the mechanically stimulated cell(s) after a combined treatment with the two drugs (*D*, representative of 5 experiments from 5 preparations). Bar = 50.0 µm.

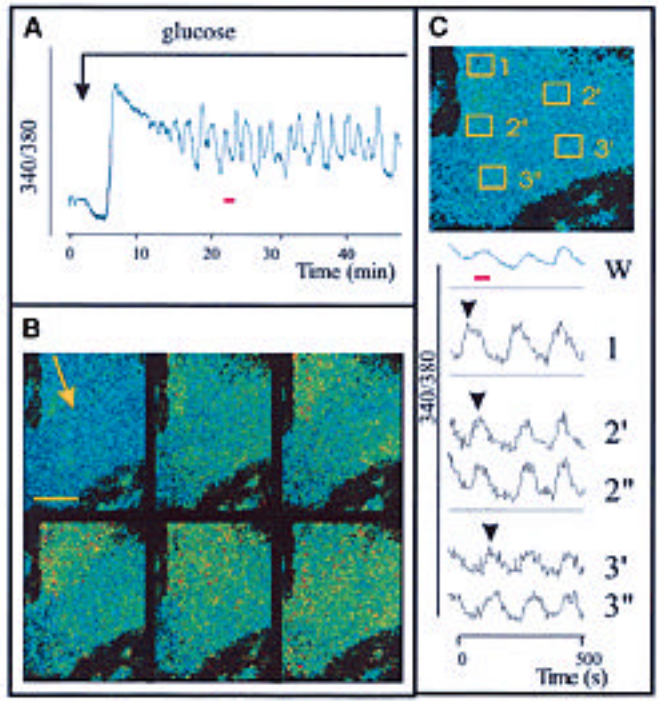

FIG. 3. $[Ca^{2+}]_i$ oscillations evoked by glucose stimulation in pancreatic islet cell monolayers. The trace in *A* illustrates the temporal plots of $[Ca^{2+}]_i$ changes after glucose stimulation in the field illustrated in *B*. The spatiotemporal aspects of the $[Ca^{2+}]_i$ oscillations marked by the red bar are further analyzed 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 the presence of 3.3 mmol/l glucose, while the following images (from top left to bottom right, 3 s apart) illustrate the evolution of the Ca^{2+} wave in response to glucose administration. The yellow arrow shows the approximate direction of the flow. The traces in *C* are recorded from the whole field (w) and from 5 different regions outlined in the upper figure. The arrows highlight the sequential $[Ca^{2+}]_i$ elevation in different regions (from 1 to 3) along the wave path and the simultaneous variation in parallel areas (i.e., 2' and 2"; 3' and 3"). Representative of 45 experiments. Bar = 50.0 µm.

strict causal connection between temporal and spatial aspects of Ca^{2+} signals under these experimental conditions, revealing that each $[Ca^{2+}]_i$ oscillation was the consequence of the propagation of a Ca^{2+} wave (Fig. 3). Noticeably, all Ca^{2+} waves progressed at a constant speed (~10 µm/sec) and in a direction approximately parallel to the stream. Variations or arrest of the perifusion did not influence the $[Ca^{2+}]_i$ in nonstimulated monolayers but deeply perturbed the oscillatory activity observed after glucose stimulation. Along with the spatiotemporal aspects of Ca^{2+} signaling, the secretory activity of β-cells was largely preserved, as documented by the secretion index, i.e., the ratio between insulin secretion peak (after 10 mmol/l glucose administration) and the preceding basal values (in presence of 3 mmol/l glucose) in single experiments. After glucose elevation, values increased severalfold under both static incubation (4.0 ± 3.0 and 15.5 ± 7.8 pg insulin/mg protein content at 3.3 and 10 mmol/l glucose, respectively; secretion index = 4.9 ± 2.7; n = 8) and perifusion (3.6 ± 2.3 and 14.8 ± 7.2 pg insulin/mg protein content at 3.3 and 10 mmol/l glucose, respectively; secretion index = 4.7 ± 1.8; n = 5).

Role of gap junctions in glucose-induced cellular responses. We next studied the effect of gap junction uncoupling on the spatiotemporal aspects of Ca^{2+} signals elicited by 10 mmol/l glucose in pancreatic islet cell monolayers. Administration of 20 µmol/l AGA blocked neither $[Ca^{2+}]_i$ oscillations nor spreading of Ca^{2+} waves (n = 13) (Fig. 4*A* and *B*); the only observed variation was a moderate change in the pattern of individual oscillations that sometimes appeared somewhat more irregular and sustained. Also, lowering the temperature up to 19°C, i.e., a condition that was reported to significantly reduce gap junctional conductance (32), did not affect the Ca^{2+} signaling pattern (n = 3) (Fig. 4*C*) even though a moderate reduction in amplitude was generally observed. A further indication that glucose-induced Ca^{2+} signals are coordinated independently of gap junctional communications was provided by the observation that correlated oscillatory patterns could be observed in monolayers physically separated from each other. These results strongly suggested that Ca^{2+} signals could travel through the extracellular space. To exclude the possibility that small cytoplasmic bridges might remain unnoticed in bright-field examination, in some experiments the surface of the coverslip was scratched with the tip of a glass microelectrode along the area of discontinuity between two monolayers. Even so, $[Ca^{2+}]_i$ oscillations showed a correlated pattern on either side, with a phase shift representing the delay as Ca^{2+} waves crossed the cleft (3 of 4 monolayers) (Fig. 5*A*).

The correlation among the responses of cells belonging to distinct monolayers was further investigated in experiments that were of comparable length (7 of the 9 experiments). Transfer function analysis was performed on selected sections of the $[Ca^{2+}]_i$ traces starting from the initial rise to peak. No models could be fitted to describe a time-independent and consistent relation between the responses of the two facing monolayers. Time-dependent cross-correlation analysis, however, pointed to a consistent pattern in the relations between the responses across the dividing cleft. Figure 5*B* is an example that illustrates the general features of correlation between responses in the areas outlined in Fig. 5*A*: the two responses were aligned on the time axis about the time of the response peak, here indicated as time 0. Correlation of the responses was very high around the initial peak of $[Ca^{2+}]_i$ and

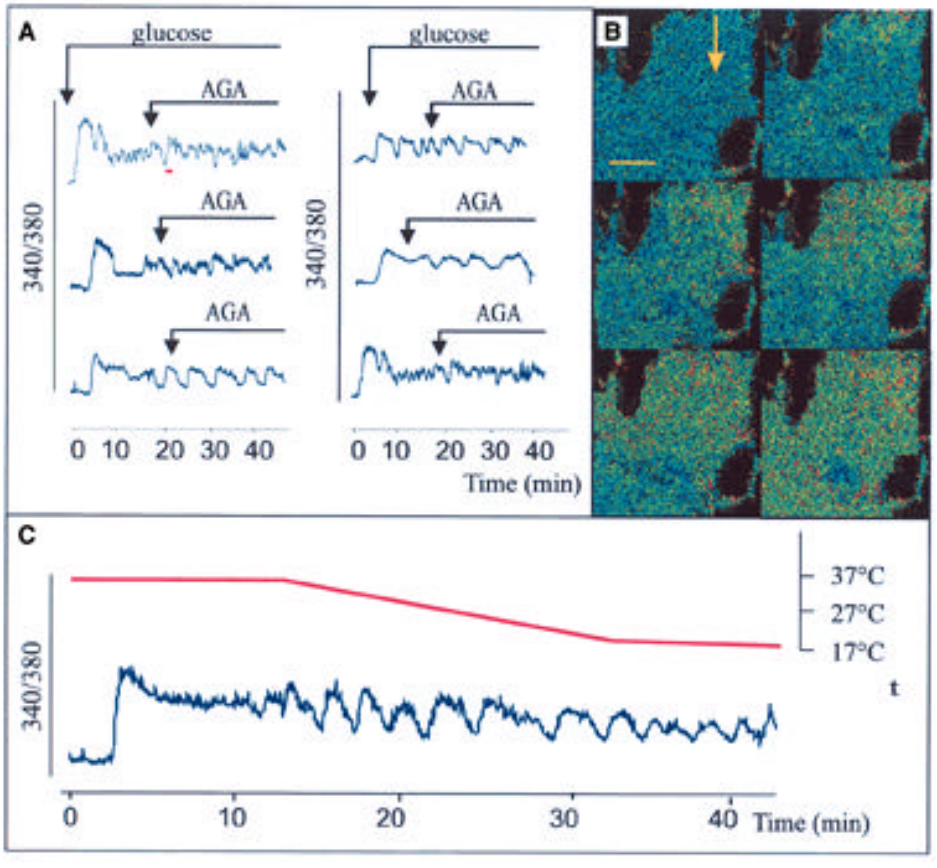

FIG. 4. Influence of gap-junctional coupling on the glucose-induced $[Ca^{2+}]_i$ changes in monolayers. *A* shows the temporal analyses of $[Ca^{2+}]_i$ in 6 different monolayers exposed to 10 mmol/l glucose (T = 0) before and after administration of 20 µmol/l AGA. The spatiotemporal aspects of the $[Ca^{2+}]_i$ oscillation marked by the red bar are reported in the gallery of images in *B*. The top left images show the $[Ca^{2+}]_i$ in 3.3 mmol/l glucose, while the following images (from top left to bottom right, 3 s apart) illustrate the evolution of the Ca^{2+} wave in response to glucose administration. The yellow arrow shows the approximate direction of the flow. Administration of the specific blocker of gap junctions inhibited neither the $[Ca^{2+}]_i$ oscillations nor the corresponding Ca^{2+} waves. Representative of 13 experiments. Bar = 50.0 µm. *C* shows the temporal analysis of glucose-induced $[Ca^{2+}]_i$ changes in a monolayer in which temperature was progressively decreased according to the upper red diagram. Even at the lowest temperature (19°C), the oscillations of $[Ca^{2+}]_i$ retained their basic pattern with only a moderate reduction in amplitude. Representative of 3 experiments.

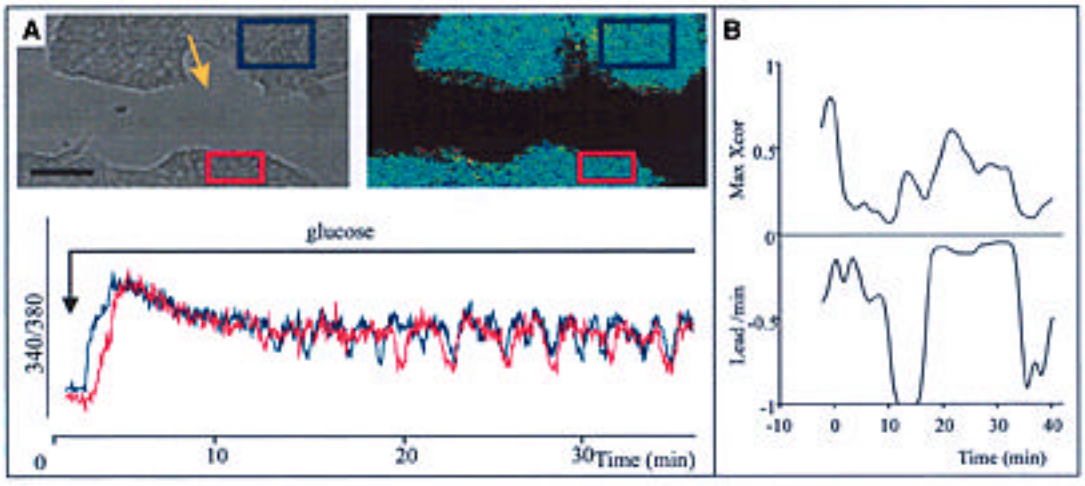

FIG. 5. Propagation of Ca^{2+} waves between separate pancreatic islet cell monolayers. The bright field image (*top left*) and the pseudocolor image (*top right*) in *A* illustrate part of two physically distinct monolayers. The yellow arrow shows the direction of the flow. To remove any possible cytoplasmic continuity between the two, a glass micropipette was drawn alongside the cell-free cleft. The red and blue traces illustrate the glucose-induced $[Ca^{2+}]_i$ changes recorded from the two areas, identified by the same colors, in the upper images. After ~20 min, the two traces displayed very similar oscillatory patterns, with the Ca^{2+} transients of the blue line generally preceding those of the red one. *B* illustrates the cross-correlation analysis performed on the traces in *A*. The two $[Ca^{2+}]_i$ signals were aligned about the peak (here indicated as time 0), and cross-correlation computation was performed on the two traces (see details in METHODS). The maximum value of the cross-correlation (for time-lags in the range 0–60 s) is plotted in the upper panel, as a function of time (the values 0 and 1 represent no and perfect correlation, respectively). The corresponding time-lag values (with changed sign) are plotted in the bottom panel. The lead of the red trace with respect to the blue one is shown, rather than the delay, to emphasize the coherence between the time courses of cross-correlation and delay (both traces were smoothed with an approximate Gaussian window of about 1.5 min duration). Bar = 50.0 µm. Representative of 9 of 13 experiments.

displayed a short delay. After the subsequent partial decay of $[Ca^{2+}]_i$, the correlation between the two signals gradually declined to very low values, the corresponding delay thereby losing any meaningfulness. Subsequently, the cross-correlation rose and remained high for about 20 min, with a relatively stable delay (~5 s). In all the examined pairs (n = 7), correlation was very high (maximum cross-correlation 0.89 ± 0.079) around the $[Ca^{2+}]_i$ peak, with variable delays (from 3 to 20 s, 10.2 ± 8.25); this finding indicates that variability in response time of each cell predominated over diffusion of the stimulating agent in determining the onset of the response. Within 15 min after the peak of the response, the correlation always vanished (maximum cross correlation 0.017 ± 0.046), the corresponding delay becoming erratic (12.6 ± 21.74 s) and thereby confirming the loss of any correlation between the two signals. Between 15 and 20 min after the peak, a new cross-correlation maximum was reached in all examined pairs (0.57 ± 0.102), with a delay between 3.5 and 21.7 s (12 ± 8.02). The high correlation was maintained for several minutes and declined in some experiments, whereas it fluctuated or remained high in others.

Finally, gap junction uncoupling of islet cells by preincubation of monolayers with AGA (20 min, 20 µmol/l) did not modify the insulin secretion index under both static incubation (4.0 ± 2.6 and 14.4 ± 7.3 pg insulin/mg protein content at 3.3 and 10 mmol/l glucose, respectively; secretion index = 4.6 ± 3.0; n = 8) and perifusion (4.3 ± 2.1 and 17.3 ± 8.8 pg insulin/mg protein content at 3.3 and 10 mmol/l glucose, respectively; secretion index = 4.0 ± 0.3; n = 5).

Therefore, evidence from both monolayers and isolated islets suggests that, on glucose stimulation, spreading of Ca^{2+} signals as well as insulin secretion might be controlled by extracellularly released factor(s) rather than by gap junctions.

The role of intracellular Ca^{2+} stores in the spreading of Ca^{2+} signaling. Two sources are potentially responsible for $[Ca^{2+}]_i$ elevation, one almost infinite, the extracellular space, and the other discrete, the intracellular Ca^{2+} stores. To evaluate the relative contributions of either source, glucose stimulation was applied in the presence of physiological concentration of extracellular Ca^{2+} (2 mmol/l), and then continued in Ca^{2+}-free medium (0 mmol/l Ca^{2+} + 0.1 mmol/l EGTA). Blockade of the oscillations ensued after Ca^{2+} deprivation (5 of 5 monolayers), and a similar result was observed after administration of 10 µmol/l nifedipine (4 of 4 monolayers) a specific blocker of voltage-dependent Ca^{2+} channels of the L type. Although we could conclude that the oscillatory activity triggered by glucose was sustained, as expected, by Ca^{2+} influx, this result did not exclude the possibility that intracellular stores might also contribute to $[Ca^{2+}]_i$ elevations. To specifically address this aspect, Ca^{2+} stores were discharged by two different pharmacological agents: Tg, the SERCA blocker, and ryanodine (Ry), an alkaloid that locks the cognate ryanodine receptors in the open configuration. Incubation with 1 µmol/l Tg, long enough (15–20 min) to eventually dissipate the steep Ca^{2+} gradient that exists between the lumen of Ca^{2+} stores and the cytosol, was usually unable to block the glucose-induced rhythmic activity (7 of 10 monolayers); in the remaining monolayers, $[Ca^{2+}]_i$ oscillations gradually subsided with the progressive elevation of $[Ca^{2+}]_i$ reaching a new, sustained plateau (not shown). In contrast, administration of 10 µmol/l Ry had no effects on $[Ca^{2+}]_i$ (5 of 5 monolayers). Similar results were reproduced in whole islets as well (not shown).

On the trail of the extracellular signal. A panel of experiments was then designed to try to identify the paracrine factor(s) able to coordinate $[Ca^{2+}]_i$ signaling within the islet. The first candidate was ATP, inasmuch as activation of

purinergic receptors can sustain the propagation of Ca^{2+} waves in the insulinoma cell line RIN (27) as well as in other cell types (23–26), not least in our monolayers, after mechanical stimulation. However, neither perifusion with a medium containing 2 U/ml apyrase (an enzyme that quickly degrades ATP, $n = 9$) nor administration of 100 µmol/l suramin (a purinergic antagonist, $n = 5$), either alone or together with 20 µmol/l AGA ($n = 3$), was able to affect the spatiotemporal patterns of glucose-induced Ca^{2+} signaling (not shown).

The participation of the following second messengers, which have been reported to influence glucose-induced insulin secretion or Ca^{2+} signaling, was also considered: arachidonic acid, investigated either by using blockers of PLA2 (10.0 µmol/l HELSS, $n = 5$; or 1.0 µmol/l wortmannin, $n = 4$) (33,34) or by inhibiting its exogenous accumulation (0.5% free fatty acid BSA added to the perifusion medium, $n = 4$); NO, by using an NO synthase inhibitor (300 µmol/l L-N-(iminoethyl)-ornithine, $n = 4$) (35); and cAMP, by administering a competitive inhibitor of cAMP-dependent protein kinase A (20 µmol/l rpcAMP, $n = 5$) (36). Finally, protein kinase C was inhibited by preincubation with 10 mmol/l Ro 31 8220 ($n = 6$) (37). None of these treatments affected glucose-induced $[Ca^{2+}]_i$ oscillations.

DISCUSSION

The propagation of Ca^{2+} signals from cell to cell, one of the basic ways to transfer information and coordinate cell functions (38), can occur via either intercellular or extracellular routes. The first take advantage of the communication provided by gap junctions to establish direct electrical and metabolic coupling among cells (14); the second convey information by releasing into the extracellular space diffusible molecules that promote $[Ca^{2+}]_i$ elevation (25–27).

In this study we exploited the property of pancreatic islet cells to grow in monolayer cultures that reproduce the organization and function of islets (39,40), to further our understanding of the mechanisms that coordinate Ca^{2+} signaling, and thus insulin secretion, in response to glucose. In previous work (16), we suggested that the appearance of glucose-induced Ca^{2+} signals in isolated islets maintained in culture might be related to the functional reconstitution of cell-to-cell interactions, and in particular of gap junctions, which are widely credited with being the principal actors in the coordination of islet activity (41).

We show here that at the time Ca^{2+} waves were able to propagate throughout the monolayers, direct cell-to-cell coupling was indeed established among cells; however, we also demonstrate that these two properties are not necessarily related. Along with the observation that Ca^{2+} waves show distinct rates of propagation under different stimulatory conditions, our results suggest that various mechanisms of Ca^{2+} signaling can operate in parallel, a condition already reported in other cell types (25–27).

Intercellular route for Ca^{2+} signal propagation. The essential role of gap junctions in the propagation of glucose-induced Ca^{2+} signals among β-cells has been repeatedly proposed but never conclusively demonstrated (42). In the present work, this issue was specifically addressed by a series of experiments in which gap junctions were blocked by AGA (30). This specific uncoupler, which has recently been reported to desynchronize intercellular $[Ca^{2+}]_i$ oscillations in various cell types (26,31), was preferred to heptanol or octanol because these two alkanols were reported to severely affect, by themselves, Ca^{2+} homeostasis (43,44). The experiments in which pancreatic islet cells were uncoupled by AGA, along with those in which gap junction conductance was reduced by lowering the temperature, demonstrate that two independent mechanisms control Ca^{2+} signal propagation. Ca^{2+} waves elicited by mechanical stimulation required functional gap junctions and the participation of intracellular Ca^{2+} stores, thus showing strong similarities to mechanisms that operate in other cells, such as astrocytes (45) and osteoblastic cells (25). With glucose stimulation, on the other hand, propagation did not require fully functional gap junctions and relied on nifedipine-sensitive Ca^{2+} influx. Since a direct correlation between glucose-induced $[Ca^{2+}]_i$ oscillations and pulsatile insulin release was reported (11,12), it was not surprising that insulin secretion was also unaffected by AGA uncoupling.

Our conclusion that physiological gap junction communication is not necessary for proper insulin secretion is in striking contrast to the proposals of other authors (9,46). Two plausible explanations can account for this discrepancy. First, previous studies were carried out using heptanol, an alkanol that suppresses Ca^{2+} currents in β-cells at concentrations lower than those required to inhibit gap junctional coupling (43). Second, the cells employed were not normal β-cells but insulin-producing tumoral cell lines that are defective in terms of both glucose responsiveness (47) and cell-to-cell coupling (9). In light of the present findings, the possibility of using tumoral cell lines and mechanical stimulation as experimental models for the study of glucose-induced events should be thoughtfully reconsidered.

Extracellular signaling sustains Ca^{2+} wave propagation in response to glucose. We cannot exclude that gap junction communication is partially maintained even after AGA treatment since, at present, there is no information on the degree of electrical coupling among pancreatic islet cells under this experimental condition. However, the coordination of Ca^{2+} waves in response to glucose is more likely controlled by the extracellular signaling route. In fact, our results suggest that one or more diffusible molecules might convey Ca^{2+} signals between physically separated monolayers and islets. The most trivial explanation, i.e., that glucose itself sequentially activates the cells along the direction of the stream, is not conceivable for at least two reasons. First, the average speed of small debris carried by the perifusion was at least one order of magnitude higher than the Ca^{2+} wave speed. Second, perifusion was constant, not pulsatile. It is difficult to imagine how spatiotemporal Ca^{2+} signals could be rhythmically reproduced without proper cellular coordination. Therefore, the presence of some diffusible factor can be postulated. Such a factor should not derive from a single cell but rather be sequentially released by the cells along the wave front of stimulation. Initially, the most likely candidate factor was ATP, a nucleotide cosecreted with insulin (48), that was reported to sustain the propagation of intercellular Ca^{2+} waves via activation of purinergic receptors in various cell types (23,25,27). We do not exclude that this mechanism has physiological relevance in β-cells; on the contrary, we provide evidence that ATP, along with gap junctions, can also sustain Ca^{2+} wave propagation after a mechanical stimulus. In the case of glucose-induced Ca^{2+} waves, however, propagation did not rely on the presence of the nucleotide and did not require

the participation of intracellular Ca^{2+} stores (22). Blockade of SERCAs, with ensuing store depletion, in fact did not induce waves but only increased in $[Ca^{2+}]_i$, an effect that is likely to be secondary to the activation of the so-called capacitative Ca^{2+} influx.

We also investigated the possible involvement of a series of signaling molecules and enzymes, such as arachidonic acid, NO, cAMP and protein kinase C, that have been reported, albeit with conflicting interpretations, to modulate stimulus-secretion coupling (49,50). However, no significant changes were noted. Numerous potential candidates remain among the glucose metabolites (21,51) or substances that are coreleased with insulin (52), even though this latter possibility seems unlikely considering that a decrease in temperature, a condition that drastically inhibits insulin secretion (53), had only marginal effects on the coordination of Ca^{2+} signals. The identification of the extracellular messenger(s) would help to clarify whether $[Ca^{2+}]_i$ oscillations occur as a result of activation of an endogenous oscillator or simply represent the link between the rhythmic delivery of some active molecule(s) and hormone release (51).

In conclusion, in this work we describe two principal mechanisms for the propagation of intercellular Ca^{2+} signaling. In the first, Ca^{2+} signal coordination is maintained by electrical or metabolic coupling via gap junctions; in the second, extracellular signaling component(s), yet to be identified, might play a key role. Our data, on the one hand, demonstrate that insulin responsiveness does not require physiologically active gap junctions and, on the other hand, raise the possibility that extracellular signal(s) may serve to synchronize the release of hormone not only in individual cells within a monolayer but also in physically distinct islets (21). The identification of soluble factor(s) able to coordinate islet cells will be of importance for the understanding of the mechanisms that underlie the recruitment of β-cells to secretion, a process that is crucial in the function of the endocrine pancreas and that has important implications in the pathophysiology of diabetes.

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