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Glucose-mediated Ca^{2+} signalling in single clonal insulin-secreting cells: evidence for a mixed model of cellular activation

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Abstract

Using clonal insulin-secreting BRIN-BD11 cells, we have assessed whether the graded response of the whole cell population to glucose can be accounted for by a dose-dependent recruitment of individual cells, an amplification of the response of the recruited cells or both. Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is an established index of β-cell function. We used fura-2 microfluorescence techniques to assess the $[Ca^{2+}]_i$ responsiveness of single BRIN-BD11 cells to glucose and other secretagogues. Glucose (1–16.7 mM) evoked oscillatory $[Ca^{2+}]_i$ rises in these cells resembling those found in parental rat pancreatic β-cells. The percentage of glucose-responsive cells was 11% at 1 mM and increased to 40–70% at 3–16.7 mM glucose, as assessed by a single-stimulation protocol. This profile was unrelated to possible differences in the cell cycle, as inferred from experiments where the cultured cells were synchronized by a double thymidine block protocol. Individual cells exhibited variable sensitivities to glucose (threshold range: 1–5 mM) and a variable dose-dependent amplification of the $[Ca^{2+}]_i$ responses (EC_{50} range: 2–10 mM), as assessed by a multiple-stimulation protocol. Glyceraldehyde and α-ketoisocaproic acid had glucose-like effects on $[Ca^{2+}]_i$. The data support a mixed model for the activation of insulin-secreting cells. Specifically, the graded secretory response of the whole cell population is likely to reflect both a recruitment of individual cells with different sensitivities to glucose and a dose-dependent amplification of the response of the recruited cells.

Keywords: Pancreatic β-cell line; Glucose metabolism; Ca^{2+} oscillations; Fura-2 fluorescence; Cellular heterogeneity

1. Introduction

It has long been known that different parameters of pancreatic β-cell function including insulin release vary with glucose concentration in

Abbreviations: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; Fura-2/AM, acetoxymethyl ester of fura-2; KIC, α-ketoisocaproic acid.

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a dose-dependent fashion. However, the physiological mechanism underlying the graded secretory response remains controversial (for revision and models see references: [1–4]). According to one model, individual β-cells are assumed to have similar sensitivities to glucose (*homogeneity* model). Alternatively, the graded response of the whole population might reflect a concentration-dependent recruitment of individual β-cells with different sensitivities to glucose (*heterogeneity* model). In vitro evidence from different sources suggests that the graded secretory response to glucose is compatible with the latter model. Indeed, isolated mouse and rat β-cells were found to be highly heterogeneous in their threshold sensitivity for glucose-induced insulin biosynthesis and release [5–11]. In contrast, whether the *homogeneity* model might also explain the graded secretory response remains to be fully determined. The difficulty in assessing the validity of this model stems, in part, from the fact that single β-cells have not been subjected to a multiple stimulation protocol in most of the latter studies. It should be noted, however, that some of the original observations by Salomon and Meda [5] and Hiriart and Ramirez-Medeles [7] might be regarded as being compatible with the *homogeneity* model.

It is well established that oscillatory rises in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$), measured from either single β-cells or pancreatic islets, are closely associated with stimulation of insulin secretion [12–16]. It is therefore possible that the observed secretory heterogeneity might reflect considerable differences in the oscillatory $[Ca^{2+}]_i$ responses, previously reported by different authors at the single cell level [17–22]. These studies had a limited potential to address the model of cellular activation underlying the heterogeneous behaviour, either because the main focus was on the response of individual β-cells to a single glucose stimulation or because they were conducted using unpurified islet cell preparations. This prompted us to assess the $[Ca^{2+}]_i$ responsiveness of single clonal β-cells (BRIN-BD11) to different glucose concentrations. BRIN-BD11 cells were recently produced by electrofusion of normal rat pancreatic β-cells with RINm5F cells [23] and present several properties suggesting that they represent an effective model of native β-cells, including a sharp sensitivity to glucose and an adequate insulin response to the sugar [23–26]. In this work we explored the common genetic background of BRIN-BD11 cells to ascertain whether the graded response to glucose might be supported by differences in the sensitivity of individual cells to the sugar.

2. Materials and methods

2.1. Cell culture and handling

The cells were cultured in RPMI medium containing 11.1 mM glucose and supplemented with 10% (v/v) heat-inactivated foetal calf serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Sigma Chemical Co, St Louis, MO, USA), as described elsewhere in detail [23]. The cells were passaged once a week and the culture medium changed every 2 days. When confluent, the cells were harvested from the culture flasks (Costar, Cambridge, MA, USA) by exposure to a versene-like solution with the following composition (mM): 135 NaCl, 2.5 KCl, 8 Na_2HPO_4, 1.5 KH_2PO_4, 0.5 EDTA and 0.004 ppm phenol red (pH 7.4). Harvested cells were either used for another culture cycle (initial density: 2.5×10^5 cells/flask) or aliquoted onto glass coverslips (2×10^4 cells/coverslip) which had been coated previously with 100 μg/ml poly-L-lysine (Sigma). The latter cells were stored in Petri dishes at 37°C in a 5% CO_2/95% air incubator, and allowed to attach to the coverslips for about 45–60 min (after which fresh culture medium was added to the Petri dishes). Cells were typically used 24–48 h after plating. The BRIN-BD11 cells used in this study were from passages 15 to 35. Cell viability, assessed by trypan blue exclusion for every batch of cells prior to the experiments, was always $>95\%$.

2.2. Synchronization of the cell cycle

In control experiments cell cycle synchronization was achieved by a double thymidine block

according to a standard procedure [27–29]. Briefly, 2 mM thymidine (Sigma) was added to the culture medium, thereby preventing the cells arriving at the S period from synthesizing DNA. After an incubation period of 12 h the whole cell population was expected to rest either at the start or intermediate stages of the S period. The thymidine block was then released for 7 h so that the cells became distributed throughout the G2, D and G1 periods. The cells were subsequently challenged with 2 mM thymidine for 12 h in order to block the cell cycle at the start of the S period, after which the block was released and the whole cell population allowed to proceed throughout the rest of the cycle in synchrony.

2.3. Solutions

The standard salt solution used in the microfluorescence experiments had the following composition (in mM): 120 NaCl, 5 KCl, 25 $NaHCO_3$, 2.56 $CaCl_2$ and 1.1 $MgCl_2$. This solution was supplemented with different glucose concentrations, as required, and constantly gassed with 95% O_2/5% CO_2 for a final pH of 7.4. The solutions used to calibrate fura-2 fluorescence in terms of $[Ca^{2+}]_i$ were as described previously [30,31].

2.4. Dye loading and microfluorometry

Cells attached to coverslips were washed in standard salt solution supplemented with 2% bovine serum albumin (Sigma). The cells were loaded with 4 μM fura-2/AM (Molecular Probes, Eugene, OR, USA) for 45 min at 37°C in this medium. The experiments were carried out using the original cell-containing coverslips as bottoms for a perifusion chamber. The temperature and flow rate in the chamber were 37°C and 1.5–2 ml/min, respectively. The $[Ca^{2+}]_i$ was recorded from single BRIN-BD11 cells using either a dual-excitation photomultiplier-based microfluorescence system (Deltascan, Photon Technology International-PTI, NJ, USA) or a dual-excitation video imaging system (Magical, Applied Imaging, Gateshead, UK), essentially as described previously for single chromaffin cells [32,33] and pancreatic β-cells [31]. Briefly, single cells were alternately excited at 340/380 nm via two monochromators (PTI system) or a stepping filter wheel (Magical system). The fluorescence was either detected by a photomultiplier in the single photon counting mode (PTI system) or by a SIT camera (Magical system) after passing through a band pass interference filter centred at 510 nm. In the case of photomultiplier-based measurements, the fluorescence ratio F_{340}/F_{380} was converted into $[Ca^{2+}]_i$ values using the calibration equation of Grynkiewicz et al. [34], as described previously by our laboratory [30–32]. The F_{340}/F_{380} ratio was used as an index of $[Ca^{2+}]_i$ for the imaging experiments.

Image acquisition and analysis were carried out as described previously in detail [33], with some modifications. Briefly, fluorescence images were acquired by averaging eight consecutive video frames at each wavelength and stored as 8-bit, 256 × 256 pixels digital images. A contour was drawn around each cell in a field and the fluorescence determined at each wavelength, after suitable correction of background fluorescence. The fluorescence ratios F_{340}/F_{380} were calculated at each time point by taking the fluorescence determined at this time point and the interpolated fluorescence for the other wavelength, in order to maximize the time resolution of the measurements. As a result of this analysis, ratio vs time plots for all cells were obtained. The fluorescence ratio images were obtained by ratioing the corresponding fluorescence images on a pixel-by-pixel basis after appropriate background correction, and were stored as 8-bit pseudo-coloured images. In protocols involving multiple stimulations photobleaching was minimized by shutting off excitation light between stimulations.

2.5. Data analysis

The glucose responsiveness of individual cells was sometimes assessed by the time integrals of the $[Ca^{2+}]_i$ signals, after subtraction of the respective background area. The latter was delimited by a straight line representing resting $[Ca^{2+}]_i$ at 1 mM glucose (or the lowest $[Ca^{2+}]_i$ level between oscillations in the case of experiments

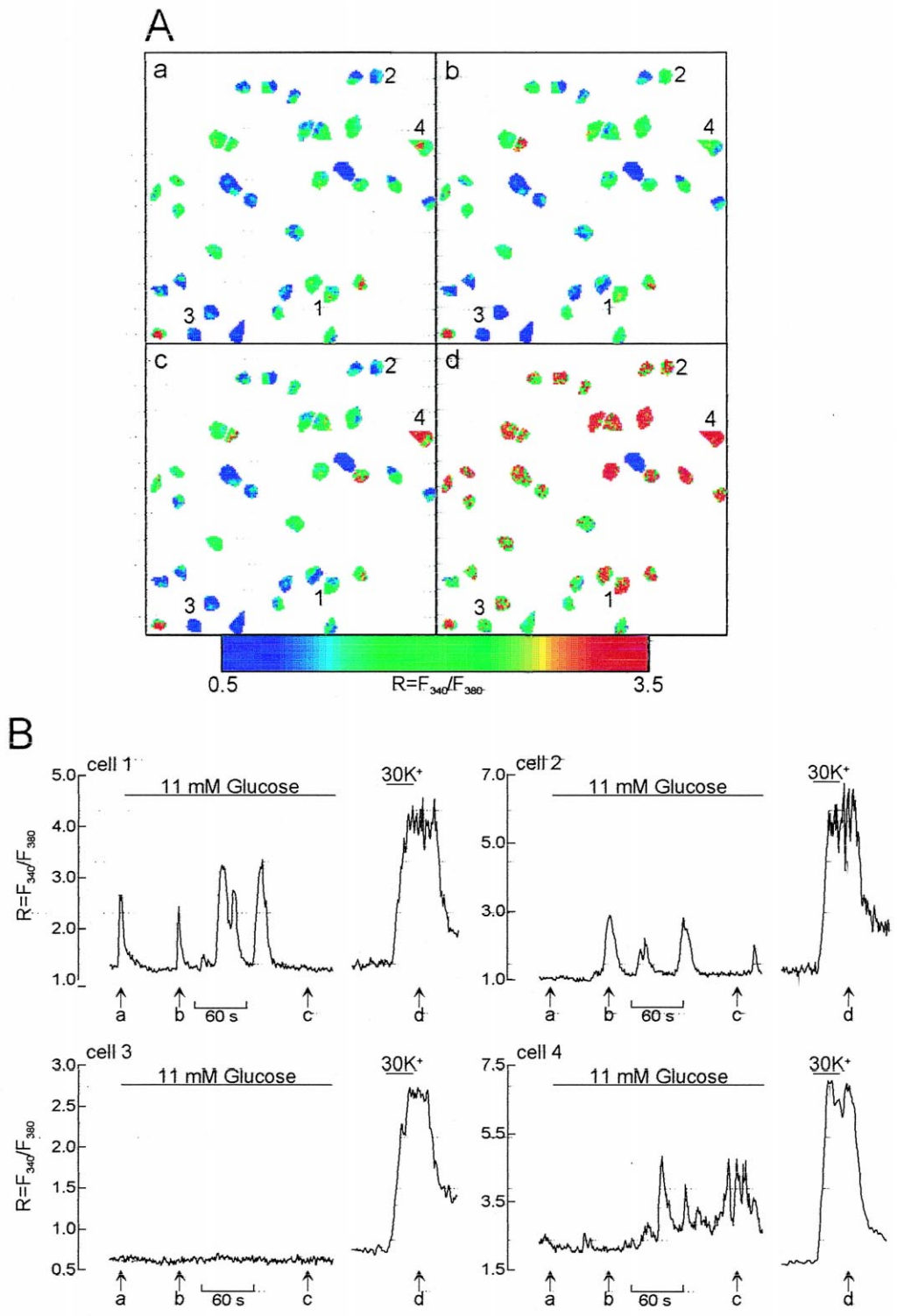

Fig. 1. (*Caption opposite*).

with spontaneous $[Ca^{2+}]_i$ activity in 1 mM glucose). Integration time for each glucose concentration was kept constant within each experiment. Integration was carried out using commercially available software (Microcal Origin).

All results (text and figures) were expressed as mean ± SEM. Statistical significance of differences between mean values was assessed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparation test, as appropriate. Differences between responses obtained at different glucose concentrations were considered to be statistically significant at the 95% confidence level.

3. Results

The $[Ca^{2+}]_i$ was recorded from single BRIN-BD11 cells using the fluorescent Ca^{2+} indicator fura-2 and either video camera (Figs. 1 and 2) or photomultiplier-based (Figs. 3 and 4) microfluorescence techniques.

3.1. Calcium imaging experiments: effects of 11 mM glucose

In the presence of 1 mM glucose (the minimal concentration used in this study), the $[Ca^{2+}]_i$ recorded from the vast majority of the cells remained low and did not undergo any spontaneous fluctuations. However, a fraction of the cells examined [ca. 11%, see Fig. 1(B) — cell 1 and Fig. 4 — cell 4 for examples] exhibited spontaneous $[Ca^{2+}]_i$ oscillations resembling those previously recorded from INS-1 cells subjected to low glucose concentrations [35,36]. This may account for the variable Ca^{2+} levels which are apparent from the video frames obtained in 1 mM glucose [Fig. 1(A-a)]: while a fraction of the cells exhibited fairly high Ca^{2+} levels, as denoted by the respective green and yellow pseudo-colours, many cells (labelled light or deep blue) actually displayed low Ca^{2+} levels typical of quiescent cells. It should be noted, however, that a few cells had fairly high Ca^{2+} levels which remained constant throughout the pre-stimulatory period [e.g. cell 4 in Fig. 1(A-a), also depicted in Fig. 1(B)].

We have investigated the $[Ca^{2+}]_i$ changes evoked by 11 mM glucose, a concentration that elicits near-maximal stimulation of insulin secretion from BRIN-BD11 cells [23]. Fig. 1(A) shows examples of video frames obtained at an early (1 min, frame b) and late stage (3.8 min, frame c) during stimulation. Glucose appeared to evoke pronounced $[Ca^{2+}]_i$ rises (denoted by green–red transitions in the pseudo-colour images) in just a small fraction of the cells (e.g. cells 2 and 4), as suggested by visual inspection of Fig. 1(A) (frames a–c). However, owing to the typical oscillatory nature of the $[Ca^{2+}]_i$ response [Fig. 1(B)], pronounced $[Ca^{2+}]_i$ rises were much more widespread and actually occurred in 72% of the cells (percentage of cells displaying $[Ca^{2+}]_i$ transients during the 4 min stimulation period).

Fig. 1(B) shows that both the amplitude and the dynamics of the oscillatory $[Ca^{2+}]_i$ responses varied widely from cell to cell, in keeping with previous observations on rat β-cells [17,18,21]. For example, the latency of the glucose responses (defined as the time elapsed from the moment of stimulation to the start of the $[Ca^{2+}]_i$ oscillations) ranged from a few tens of seconds in rare cases to 1–2 min in the vast majority of the

Fig. 1. Video imaging analysis of the $[Ca^{2+}]_i$ responses of BRIN-BD11 cells to 11 mM glucose. (A) Pseudo-colour images of the ratio of fura-2 fluorescence (R) at 340 and 380 nm. Following a resting period in 1 mM glucose (frame a) the cells were stimulated with 11 mM glucose (frames b and c), allowed to recover for 15 min in 1 mM glucose (not shown) and finally challenged with 30 mM KCl (frame d). Frames b and c were obtained 1 and 3.8 min after raising glucose concentration to 11 mM. Fura-2 fluorescence ratios are assigned to different colours using the code bar at the bottom of panel A (dark blue, indicative of low $[Ca^{2+}]_i$: $R \approx 0.5$; red, indicative of high $[Ca^{2+}]_i$: $R \approx 3.5$). (B) Dynamics of $[Ca^{2+}]_i$ changes evoked by glucose and KCl depolarization in four identified BRIN-BD11 cells (cells 1–4 in the different frames of panel A). The bars denote the periods of stimulation with 11 mM glucose and 30 mM KCl (30 K^+). Basal glucose concentration was 1 mM. Arrows labeled a–d correspond to the different frames of panel A.

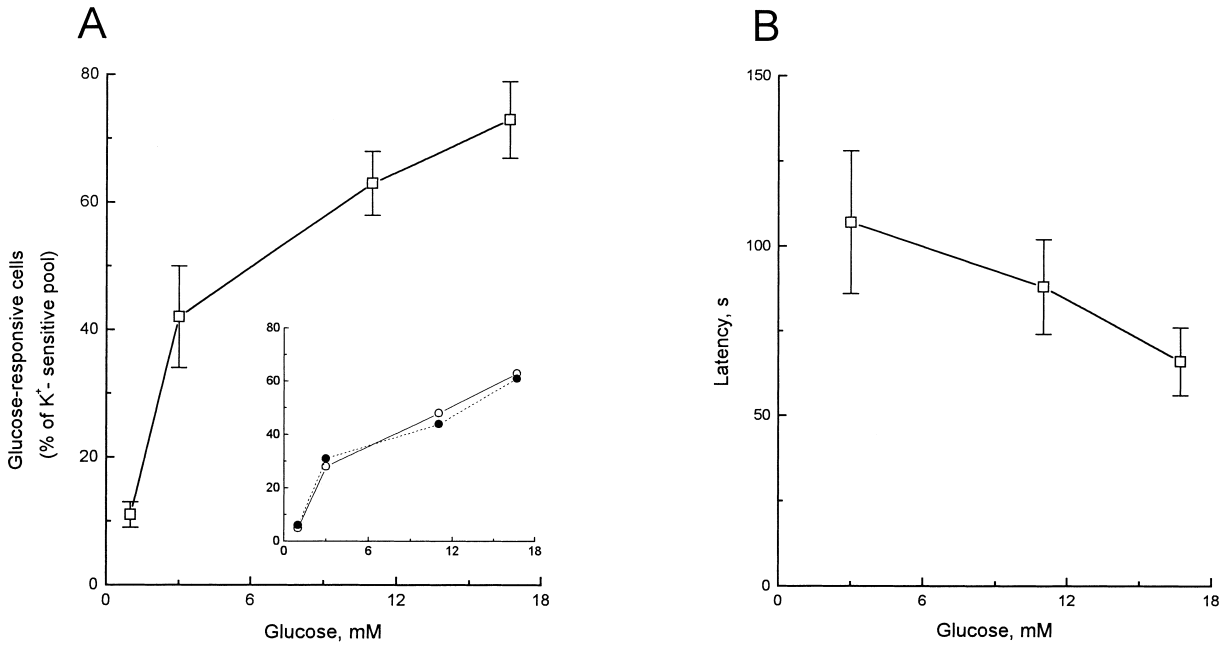

Fig. 2. Dose-dependent activation of BRIN-BD11 cells by glucose. (A) Percentage of glucose-responsive cells (cells exhibiting an oscillatory $[Ca^{2+}]_i$ response to glucose) vs glucose concentration. For each glucose concentration approximately 95% of the cells displayed $[Ca^{2+}]_i$ rises when stimulated with 30 mM KCl. This pool of K^+-sensitive cells (n = 93–139) was taken as 100%. Insert: percentage of glucose-responsive cells vs glucose concentration for 6 control experiments (2 experiments for each glucose concentration in the range 3–16.7 mM) where the cells (n = 29–41) were subjected to a double thymidine block protocol (solid circles). (B) Time elapsed from the moment of stimulation to the start of the $[Ca^{2+}]_i$ oscillations (latency) vs glucose concentration (n = 39–85 cells). Data are presented as mean ± SEM. Data from the experiment depicted in Fig. 1 and 11 similar experiments (4 experiments for each glucose concentration in the range 3–16.7 mM).

cells examined (e.g. cells 2 and 4). It is also noteworthy that a fraction of the cells (28% for the experiment depicted in Fig. 1, e.g. cell 3) did not respond to 11 mM glucose. This is unlikely to be accounted for by lack of functional voltage-sensitive Ca^{2+} channels, inasmuch as the vast majority of the latter cells retained the ability to respond to a depolarizing stimulus (exposure to brief pulses of 30 mM KCl) with pronounced $[Ca^{2+}]_i$ rises [Fig. 1(A-d) and example in Fig. 1(B), cell 3]. Indeed, high K^+ evoked $[Ca^{2+}]_i$ rises in approximately 95% of the cells examined, as indicated by the widespread green–red transitions in the pseudo-colour images of Fig. 1(A-d). It seems therefore that either glucose metabolism is compromised within the glucose-insensitive fraction or that individual cells might have different sensitivities to the hexose, with 11 mM glucose being insufficient to activate some of the cells.

3.2. Dose-dependent effects of glucose on $[Ca^{2+}]_i$

We have assessed the glucose responsiveness of BRIN-BD11 cells at different concentrations in the range 3–16.7 mM. The protocol in these experiments was identical to that used in the experiments depicted in Fig. 1 (single stimulation protocol): glucose concentration was raised from 1 to either 3, 11 or 16.7 mM for 4–5 min periods, and subsequently lowered to 1 mM for about 15 min, after which the cells were stimulated with brief pulses of high K^+ solutions.

Based on the time course data generated for each cell, we computed the percentage of cells that exhibited an oscillatory $[Ca^{2+}]_i$ response (i.e.

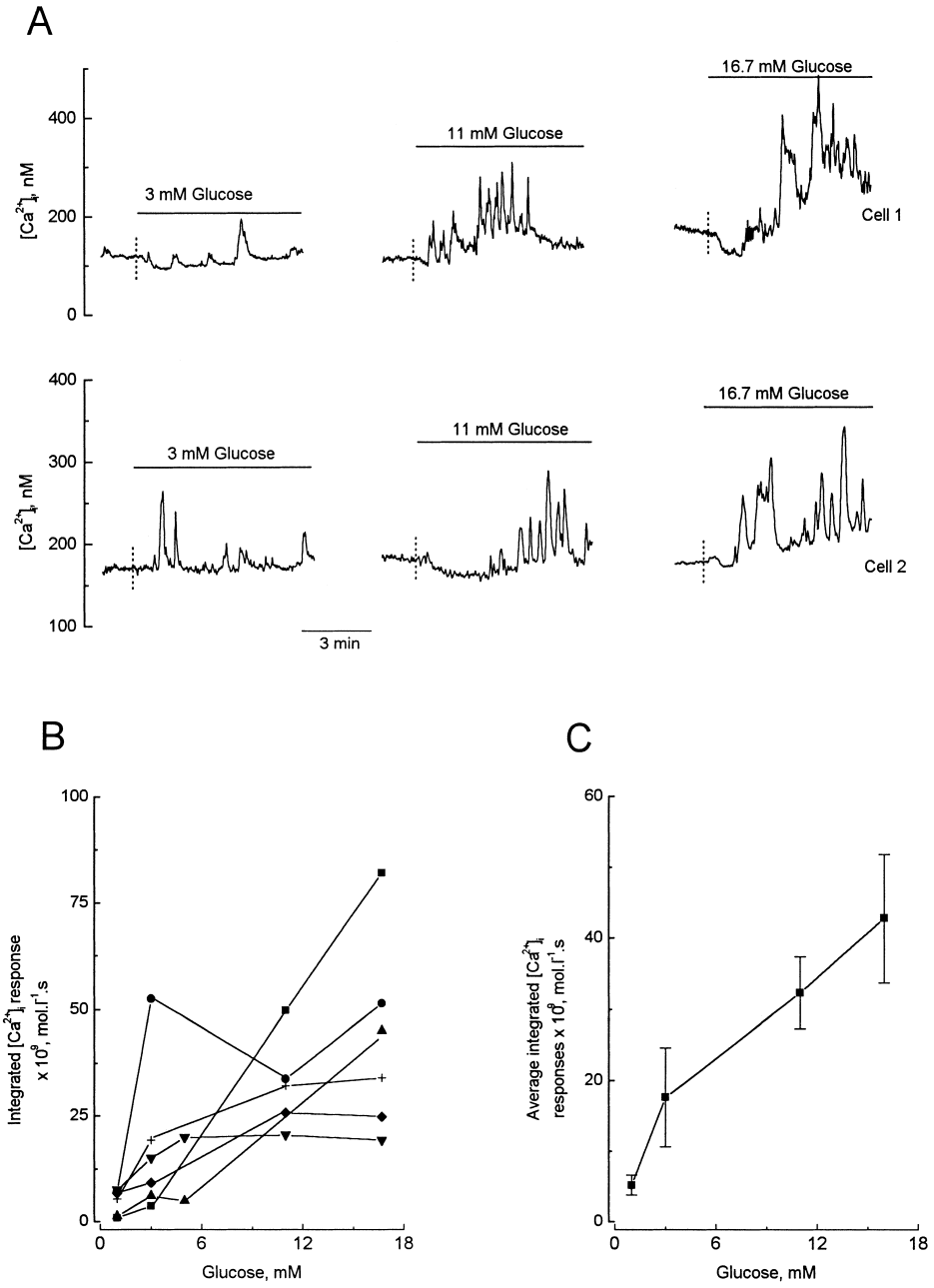

Fig. 3. Effect of increasing glucose concentrations on the $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ was recorded from single BRIN-BD11 cells by a photomultiplier-based microfluorescence procedure (ratiometric fura-2 microfluorometry). (A) Each cell was sequentially challenged with 3, 11 and 16.7 mM glucose pulses, as indicated by the horizontal bars. Cells were allowed to recover in 1 mM glucose for 15 min between stimulations. (B) Time integral of the $[Ca^{2+}]_i$ responses as a function of glucose concentration. The integrals were corrected for the respective background areas (see Section 2). Data from cells 1 and 2 in panel A (squares and crosses, respectively) and four other similar experiments. (C) Average time integral of the $[Ca^{2+}]_i$ response as a function of glucose concentration. Data are presented as mean ± SEM ($n = 6$ cells). The difference between data in 1 and 16.7 mM glucose was statistically significant ($P < 0.01$). Differences between data obtained in 3 or 11 mM and 16.7 mM glucose failed to reach statistical significance ($P > 0.05$).

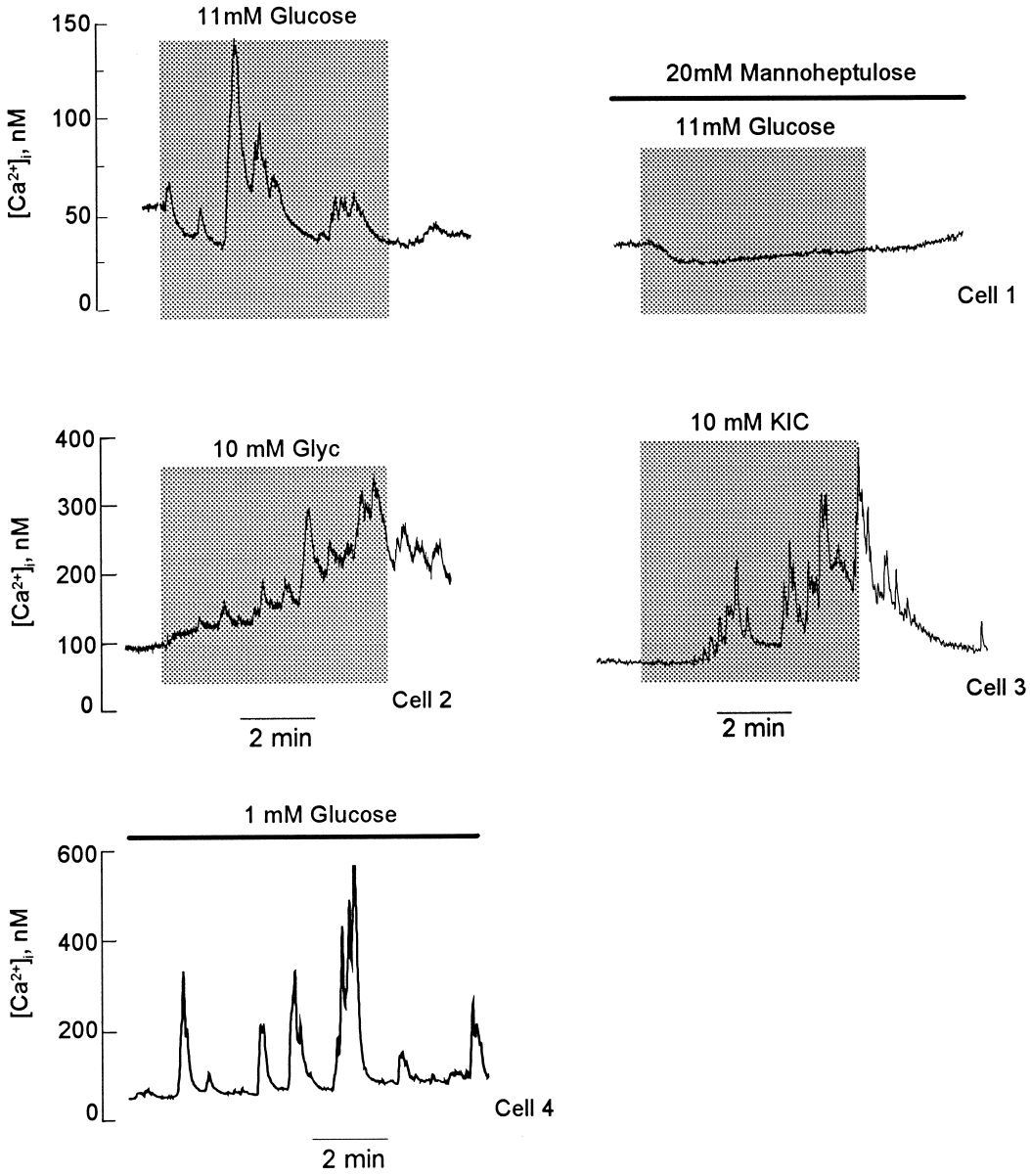

Fig. 4. Responses to metabolic stimuli and inhibitor. The $[Ca^{2+}]_i$ was recorded from single BRIN-BD11 cells as for the experiments depicted in Fig. 3. Cell 1 (representative of 3 experiments) was challenged twice with 11 mM glucose, first in the absence (left) and 15 min later in the presence of 20 mM mannoheptulose. Time in mannoheptulose prior to glucose stimulation was 10 min. Cells 2 (representative of 3 experiments) and 3 (representative of 6 experiments) were challenged with 10 mM glyceraldehyde (Glyc) and 10 mM α-ketoisocaproic acid (KIC), respectively, as indicated by the shadowed boxes. Basal glucose concentration was 1 mM (all experiments). Cell 4 is an example of a cell with spontaneous $[Ca^{2+}]_i$ oscillations in 1 mM glucose.

the percentage of glucose-responsive cells) at different glucose concentrations. For this analysis 100% was taken as the entire pool of K^+ (30 mM)-sensitive cells (K^+-insensitive cells, which constituted less than 5% of the total population, were assumed to be either nonviable or to lack functional voltage-sensitive Ca^{2+} channels and, thus, to be incapable of yielding positive responses to glucose). Fig. 2(A) shows that the percentage of glucose-responsive cells increased progressively with glucose concentration in the range 1–16.7 mM ($P < 0.0001$). Importantly, raising glucose concentration from 1 to 3 mM increased the fraction of the glucose-responsive cells to approx. 42%. A further stimulation with 11 and 16.7 mM glucose increased the size of this pool to 63 and 73%, respectively ($P < 0.05$).

It is also noteworthy that about 27% of the cells did not respond to 16.7 mM glucose, a concentration for which glucose-induced insulin release from BRIN-BD11 cells is already maximal [23,26]. It could be argued that the latencies of the glucose responses might exceed the glucose stimulation periods used in the experiments (4–5 min). This is highly unlikely, however, since a frequency distribution analysis of the latencies found in 16.7 mM glucose revealed a skewed distribution with 45 and 96% of the data points lying below 50 and 200 s, respectively (data not shown; $n = 79$ cells). A similar analysis, carried out for 11 mM glucose, revealed that 94% of the data points lay below 200 s ($n = 85$ cells). We have also computed the average latencies of the glucose responses at the different concentrations. These latencies showed a tendency to fall (not statistically significant, $P > 0.05$) with glucose concentration in the range 3–16.7 mM [Fig. 2(B)].

It could be argued that the concentration dependency of the fraction of glucose-responsive cells [Fig. 2(A)] might somehow be linked to the stage of the cell cycle. In order to assess this possibility we have carried out similar experiments involving cells subjected to the double thymidine block protocol (see Section 2). As illustrated in the insert to Fig. 2(A), the fraction of glucose-responsive cells within the synchronized cell population (solid circles) did not differ from that in control.

Fig. 2(A) suggests that the graded response of the whole cell population is accounted for by a dose-dependent recruitment of individual cells by glucose. It is also possible, however, that the response of individual cells might be amplified in a dose-dependent fashion. To assess this possibility, we have carried out experiments (multiple stimulation protocol) whereby each cell was sequentially subjected to glucose pulses with different amplitudes (range: 3–16.7 mM; basal glucose concentration: 1 mM). In these experiments the cells were allowed to recover in 1 mM glucose for about 15 min between consecutive stimulations. To minimize fura-2 photobleaching the $[Ca^{2+}]_i$ was recorded by a photomultiplier-based microfluorescence procedure and the excitation light was shut off between stimulations (see Section 2).

The effects of increasing glucose concentration on $[Ca^{2+}]_i$ are illustrated in Fig. 3(A) for two different cells. Both cells exhibited a graded response to glucose throughout the whole concentration range, as evaluated by both the maximal amplitude of the $[Ca^{2+}]_i$ transients and the time spent at elevated $[Ca^{2+}]_i$ levels. We have calculated the time integral of the $[Ca^{2+}]_i$ signal as an index of glucose responsiveness (see Section 2). The integrated responses for the different experiments are plotted in Fig. 3(B) as a function of glucose concentration. This analysis reveals that, for most cells, the integrated $[Ca^{2+}]_i$ response varied dose dependently in the range 1–16.7 mM. In these experiments the threshold for glucose-evoked $[Ca^{2+}]_i$ changes (defined as the minimal glucose concentration required for $[Ca^{2+}]_i$ activity) and the approximate EC_{50} value (defined as the glucose concentration producing half of the maximal integrated $[Ca^{2+}]_i$ response) fell in the range 1–5 mM and 2–10 mM, respectively. On average, the integrated $[Ca^{2+}]_i$ response appeared to increase with glucose-concentration in a dose-dependent fashion [Fig. 3(C); average $EC_{50} = 6.5$ mM]. Interestingly, the glucose dependency of the integrated $[Ca^{2+}]_i$ response depicted in Fig. 3(C) is similar to the variation of insulin output as a function of glucose concentration, as obtained from BRIN-BD11 cell populations subjected to either 20 min (Salgado et al., unpub-

lished observations) or 45 min [26] static incubation periods.

We have carried out control experiments whereby each cell was repetitively stimulated with a constant glucose concentration (11 mM). The integrated $[Ca^{2+}]_i$ response for each glucose pulse did not vary to any significant extent throughout the experiment (2 experiments, data not shown).

3.3. Effects of other metabolic stimuli on $[Ca^{2+}]_i$

We have carried out experiments to demonstrate the metabolic origin of the glucose-evoked $[Ca^{2+}]_i$ responses in BRIN-BD11 cells. Fig. 4 (cell 1) shows that mannoheptulose, a glucokinase inhibitor [37], suppressed the glucose-induced $[Ca^{2+}]_i$ rise observed in control (first glucose pulse). As for native pancreatic β-cells [38] stimulation with the glycolitic intermediate glyceraldehyde produced $[Ca^{2+}]_i$ rises resembling those evoked by glucose (cell 2). α-Ketoisocaproic acid (KIC), a metabolic substrate that feeds directly the mitochondria [39] produced glucose-like effects on BRIN-BD11 cells (cell 3).

4. Discussion

Two different models (*homogeneity* vs *heterogeneity*) have been proposed to explain the graded responses to glucose displayed by pancreatic β-cells [1–4]. Previous studies on isolated rat and mouse β-cells have shown that glucose stimulation leads to a dose-dependent recruitment of individual cells, therefore supporting the *heterogeneity* model. Indeed, individual β-cells have been reported to differ in the glucose threshold levels for pro-insulin biosynthesis [9], glucose metabolism [9,10,17,40], $[Ca^{2+}]_i$ [17,40,41] and secretory activity [5,7,8,11]. It should be noted, however, that owing to methodological constraints and to the lack of multiple stimulation protocols for a given cell under study most of these reports did not provide an adequate assessment of the *homogeneity* model. Using ultra-sensitive microfluorescence techniques and a combination of glucose stimulation protocols, we have been able in this study to assess both the single cell thresholds and dose-dependency profiles for glucose-induced $[Ca^{2+}]_i$ rises. We found that glucose induced a dose-dependent recruitment of individual cells [Fig. 2(A)], in keeping with the *heterogeneity* model. Importantly, however, we also found that single cells exhibited a concentration-dependent enhancement of their $[Ca^{2+}]_i$ responses (Fig. 3), thus providing evidence for an essential characteristic of the *homogeneity* model. Thus, our study lends support to a mixed model, as already suggested by others from the standpoint of insulin secretion [5,7].

The functional heterogeneity reported by others is substantially preserved following tissue culture and is therefore unlikely to represent mere variations due to metabolic status of donor animals, variable cellular damage due to enzyme/chemical isolation or different cellular contacts with α- or δ-cells which vary in both number and distribution in islets [42,43]. Similarly, the functional heterogeneity reported in our study is likely to be a largely inherent trait in BRIN-BD11 cells and other clonal cell lines which have the apparent advantage of being derived from a common genetic background and being exposed to carefully controlled homogeneous environmental conditions. It could nonetheless be argued that such cell lines do not exactly mirror the function of normal β-cells and that possible cell cycle differences amongst the cell population may play some role in the observed $[Ca^{2+}]_i$ heterogeneity. This is unlikely to be the case, however, since cell populations which have been synchronized by thymidine exhibited a glucose sensitivity similar to that observed in control (this work).

The mechanisms underlying functional heterogeneity among pancreatic β-cells are not known, but some evidence suggests a close correlation between heterogeneous behaviour and intercellular differences in glucose catabolism, possibly related to intercellular differences in hexokinase isoenzyme expression and activity [3,4]. An heterogeneous pattern of glucokinase staining in islet tissue [44] is indeed consistent with this possibility. This is also an attractive hypothesis to explain the functional heterogeneity among in-

dividual BRIN-BD11 cells. Another hypothesis to account for the heterogeneity of the observed $[Ca^{2+}]_i$ responses is the existence of individual differences in the size of the ATP-sensitive K^+ (K-ATP) channel current [45]. (Physiological release of insulin is the result of a complex sequence of events, including metabolic degradation of glucose to yield ATP, inhibition of K-ATP channels following a rise in the cytosolic ATP/ADP ratio, membrane depolarization, activation of voltage-sensitive Ca^{2+} channels, rise in $[Ca^{2+}]_i$ and subsequent processes leading to exocytosis [46–48].) Importantly, the rather uniform character of the $[Ca^{2+}]_i$ responses of individual cells to high K^+ depolarization (Fig. 1) suggests that the heterogeneity of the glucose response stems from effects that the sugar has prior to opening voltage-sensitive Ca^{2+} channels. It is noteworthy that approx. 25% of the cells in our study did not respond to supra-maximal glucose concentrations while displaying a normal response to high K^+ depolarization. This suggests that the glucose-insensitive cells either lack appropriate metabolic signals or lack a critical density of functional K-ATP channels to trigger depolarization and Ca^{2+} influx.

We have also shown that $[Ca^{2+}]_i$ rises in BRIN-BD11 cells can be evoked by demonstrated metabolic activators of pancreatic β-cells (Fig. 4). This reinforces the view that, similar to native β-cells, both glycolitic and mitochondrial metabolism are an integral part of the glucose response in BRIN-BD11 cells [25]. Thus, stimulus-secretion coupling in these cells appears to be similar to that in normal pancreatic β-cells, as pointed out by different authors. Indeed, BRIN-BD11 cells express the Glut-2 glucose transporter, have a high glucokinase/hexokinase ratio typical of native β-cells and have a $[Ca^{2+}]_i$ responsiveness similar to that of native β-cells [23,24,26]. We note however that the fraction of glucose-responsive BRIN-BD11 cells was half-maximal at ca 2–3 mM glucose [Fig. 2(A)]. This may be taken to indicate that the glucose dose–response curves in these cells are somewhat shifted towards lower glucose concentrations compared to the parental normal rat pancreatic β-cells, whose EC_{50} values for glucose-evoked $[Ca^{2+}]_i$ rises and insulin release lie in the range 5–7 mM [7,8,11,17].

This study emphasizes the utility of BRIN-BD11 cells, and other possible future β-cell lines generated by electrofusion, for further studies on the mechanisms underlying insulin stimulus-secretion coupling. Most importantly, however, we have shown that individual clonal β-cells differ markedly in their threshold sensitivity of the glucose-induced calcium activity. This, together with the dose-dependent amplification of single cell responses, may provide a basis for the graded response of insulin-secreting cell populations including whole pancreatic islets.

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