Measurements of Cytoplasmic Ca^{2+} in Islet Cell Clusters Show That Glucose Rapidly Recruits β-Cells and Gradually Increases the Individual Cell Response

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The proportion of isolated single β-cells developing a metabolic, biosynthetic, or secretory response increases with glucose concentration (recruitment). It is unclear whether recruitment persists in situ when β-cells are coupled. We therefore measured the cytoplasmic free Ca^{2+} correction ($[Ca^{2+}]_i$) (the triggering signal of glucose-induced insulin secretion) in mouse islet single cells or clusters cultured for 1–2 days. In single cells, the threshold glucose concentration ranged between 6 and 10 mmol/l, at which concentration a maximum of ~65% responsive cells was reached. Only 13% of the cells did not respond to glucose plus tolbutamide. The proportion of clusters showing a $[Ca^{2+}]_i$ rise increased from ~20 to 95% between 6 and 10 mmol/l glucose, indicating that the threshold sensitivity to glucose differs between clusters. Within responsive clusters, 75% of the cells were active at 6 mmol/l glucose and 95–100% at 8–10 mmol/l glucose, indicating that individual cell recruitment is not prominent within clusters; in clusters responding to glucose, all or almost all cells participated in the response. Independently of cell recruitment, glucose gradually augmented the magnitude of the average $[Ca^{2+}]_i$ rise in individual cells, whether isolated or associated in clusters. When insulin secretion was measured simultaneously with $[Ca^{2+}]_i$, a good temporal and quantitative correlation was found between both events. However, β-cell recruitment was maximal at 10 mmol/l glucose, whereas insulin secretion increased up to 15–20 mmol/l glucose. In conclusion, β-cell recruitment by glucose can occur at the stage of the $[Ca^{2+}]_i$ response. However, this type of recruitment is restricted to a narrow range of glucose concentrations, particularly when β-cell association decreases the heterogeneity of the responses. Glucose-induced insulin secretion by islets, therefore, cannot entirely be ascribed to recruitment of β-cells to generate a $[Ca^{2+}]_i$ response. Modulation of the amplitude of the $[Ca^{2+}]_i$ response and of the action of Ca^{2+} on exocytosis (amplifying actions of glucose) may be more important.
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The control of insulin secretion by glucose involves two major pathways that both require metabolism of the sugar by β-cells (1). The triggering pathway serves to raise the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$), which stimulates exocytosis of insulin granules. This rise essentially depends on the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels activated by a membrane depolarization that is underlain by closure of ATP-sensitive K^+ channels (2–4). The amplifying pathway, which does not imply changes in the activity of ATP-sensitive K^+ channels and in $[Ca^{2+}]_i$, serves to produce as yet incompletely identified signals that augment the action of Ca^{2+} on exocytosis (5–9).

The glucose dependency of insulin secretion and many other events occurring in β-cells is characterized by a sigmoidal relationship. This type of response may result from an increase in the individual response of functionally homogeneous β-cells, from the progressive recruitment into an active state of β-cells with distinct glucose sensitivities, or both (10).

Isolated β-cells differ in their individual sensitivity to glucose. Measurements of insulin secretion (11–14), insulin biosynthesis (15,16), and glucose metabolism (17,18) have shown a large heterogeneity in the glucose responsiveness of single β-cells. The threshold glucose concentration is variable, hence the percentage of cells developing a functional response increases with the glucose concentration.

Physiologically, β-cells are not isolated but associated within the islets of Langerhans, where intercellular coupling or paracrine influences may erase their individual differences to constitute a functionally homogeneous population. Thus, in contrast to the heterogeneous responses produced in isolated β-cells, glucose induced a uniform increase in NAD(P)H autofluorescence in β-cells residing within intact islets (19). However, studies of the nucleus size (20), of the insulin gene promoter activity (21), of protein synthesis (16), and of the rough endoplasmic reticulum size (22) suggest that some degree of β-cell heterogeneity persists in situ. There also exist differences in the threshold for glucose-induced electrical activity in β-cells within islets, but the range is limited to ~5.5–11 mmol/l glucose (23–25), and interislet variability partly accounts for these differences.

Whether glucose-induced recruitment of β-cells into an active secretory state persists or is abolished when the cells are coupled is still unresolved. Sustained stimulation of insulin secretion in vivo by hours of hyperglycemia or

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$[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration.

by glibenclamide revealed differences in the degree of β-cell degranulation within individual islets—a picture that is compatible with heterogeneity of secretion (26). Unfortunately, the question is difficult to address directly in vitro because no current technique permits measurements of insulin secretion from individual cells within islets or clusters. The $[Ca^{2+}]_i$ rise is the most important event that can be monitored to identify β-cells stimulated to secrete amidst inert but associated cells.

In this study, therefore, we characterized the effects of increasing concentrations of glucose on $[Ca^{2+}]_i$ in small clusters of mouse islet cells to determine whether β-cells are progressively recruited to produce the signal triggering insulin secretion. We compared these effects with those in islet single cells and correlated them with the changes in insulin secretion.

RESEARCH DESIGN AND METHODS

Solutions. The control medium used for islet isolation and for the experiments was a bicarbonate-buffered solution that contained (in mmol/l) 120 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, and 24 $NaHCO_3$. It was gassed with O_2-CO_2 (94:6) to maintain pH 7.4 and was supplemented with 0.5 mg/ml bovine serum albumin (fraction V). The Ca^{2+}-free solution used to disperse islets in isolated cells and clusters contained (in mmol/l) 138 NaCl, 5.6 KCl, 1.2 $MgCl_2$, 5 HEPES, and 1 EGTA, with 100 IU/ml penicillin and 100 μg/ml streptomycin, and its pH was adjusted to 7.35 with NaOH. The medium used for cultures was RPMI 1640 medium containing 10 mmol/l glucose (except in experimental series 2, in which 7 mmol/l glucose was used), 2 mmol/l glutamine, 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Preparation. Islets were aseptically isolated by collagenase digestion of the pancreas of fed female NMRI mice, followed by hand selection (27). To obtain isolated cells and clusters, the islets were incubated for 5 min in a Ca^{2+}-free solution. After centrifugation, this solution was replaced by culture medium, and the islets were disrupted by gentle pipetting through a siliconized glass pipette. Clusters and isolated cells were then cultured for 1 or 2 days on 22-mm circular glass coverslips (28).

Experimental series. Four independent series of experiments were performed. In the first series, we compared the effects of a wide range of glucose concentrations (6–20 mmol/l) on $[Ca^{2+}]_i$ changes in islet single cells and clusters of 2–20 cells from the same preparations (same coverslips). The second series was similar except for the glucose concentration in the culture medium (7 instead of 10 mmol/l). In the third series, we investigated the characteristics of $[Ca^{2+}]_i$ changes in the different cells of selected clusters. Because one cell showing a $[Ca^{2+}]_i$ change in a cluster may mask the presence of a nonresponding cell located above or below, clusters of 2–15 cells forming monolayers (no superimposed nuclei) were selected. In the fourth series, we directly compared the changes in $[Ca^{2+}]_i$ and insulin secretion in the same preparations (same coverslip).

Measurements of $[Ca^{2+}]_i$. Clusters and cells attached to the coverslips were loaded for 60 min with fura-2 (series 3) or for 90 min with fura-PE3 (series 1, 2, and 3) in control medium containing 10 mmol/l glucose and 1 μmol/l fura-2 or fura-PE3 acetoxymethylester. The coverslip was then transferred into a temperature-controlled perifusion chamber (Intracell; Royston, Herts, U.K.) of which it formed the bottom. The chamber was placed on the stage of an inverted microscope (40× objective) and perifused (1.5 ml/min) at 37°C with control medium containing increasing glucose concentrations. Cells and clusters were successively excited at 340 and 380 nm, and the fluorescence emitted at 510 nm was captured by a CCD camera (Photonic Science, Turnbridge Wells, U.K.). The images were analyzed by the MagiCal system (Applied Imaging, Sunderland, U.K.). The intervals between successive $[Ca^{2+}]_i$ measurements (ratios of the 340- and 380-nm images) were 2.4 s for the experiments lasting 40 min (series 3) and 4.8 s for the longer experiments (series 1, 2, and 3). Other details of the technique, including the method for in vitro calibration of the signals, can be found elsewhere (28,29).

At the end of each experiment, the perifusion was stopped and the chamber was filled with 1 ml phosphate-buffered saline containing 75 μmol/l propidium iodide and 0.67 μmol/l acridine orange (Sigma, St. Louis, MO) during 5 min (30). Excitation at 490 nm and reading of the emitted fluorescence at 510 nm visualizes living cells in green and dead cells in red. After the test of cell viability, the chamber was filled with 1 ml of control solution containing 1 μmol/l bisbenzimide (Sigma) for 30 min. The preparation was excited at 365 nm to reveal fluorescent nuclei, permitting unambiguous identification of single cells and counting of the number of cells within clusters.

Combined measurements of insulin secretion and $[Ca^{2+}]_i$. Clusters and isolated cells cultured for 2 days on a coverslip were loaded with fura PE3 before being transferred into the recording system, as described above, except that a 20× objective was used. The chamber was then perfused at a flow rate of 1.5 ml/min, and the effluent was collected in 2-min fractions that were immediately centrifuged to eliminate cells detached from the preparation. Insulin was measured in duplicate 400-μl aliquots of each fraction. The characteristics of the assay have previously been reported (27). It should be borne in mind that $[Ca^{2+}]_i$ was measured over a window covering <0.1% of the coverslip area. The $[Ca^{2+}]_i$ signal was thus representative of the changes occurring in the many more cells and clusters from which insulin secretion was measured. The possible presence of dead cells and the number of cells in the observed field were not evaluated in this series.

Immunodetection of somatostatin and glucagon cells. To determine the proportion of non–β-cells in the preparations, coverslips with cells and clusters cultured for 2 days were fixed in Bouin Allen's fluid (European Laboratory Supplies, Bienvere, Belgium) during 6 h at room temperature. They were then processed to immunostain α- and δ-cells with a mixture of antiglucagon and antisomatostatin serum, each at a dilution of 1:25,000 (Novo Biolabs, Bagsvaerd, Denmark). Positive cells were identified by a peroxidase method using 3,3-diaminobenzidine as the substrate for staining. The preparations were then counterstained with hemalun. The method has been described in full elsewhere (31). Labeled non–β-cells were counted for five different cultures, and their proportion was determined by counting the number of nuclei.

Presentation of results. The experiments are illustrated by representative recordings, and quantified data are presented as means ± SE.

RESULTS

Cellular composition of the preparations. On average, the cell populations attached to the coverslips comprised 15% single cells and 85% of cells within clusters of different sizes. Preparations from five different cultures were immunostained with a mixture of antiglucagon and antisomatostatin serum. The proportion of non–β-cells was 13 ± 1.4% in the whole preparations but was higher among isolated cells (33 ± 3.8%) than within clusters (9 ± 1.1%), of which 58% did not contain non–β-cells. However, the probability that single non–β-cells were studied is less because of our selection of fields containing relatively large cells. Mouse β-cells are larger than α- and δ-cells (32). The clusters used for the experiments of series 1–3 were selected on their size, which comprised between 2 and a maximum of 15–20 cells (mean 7.2 ± 0.3 cells, n = 220).

Influence of increasing glucose concentrations on $[Ca^{2+}]_i$ in islet single cells and clusters. Islet single cells and clusters cultured for 1–2 days in the presence of 10 mmol/l glucose were stimulated by stepwise increases in the glucose concentration while their $[Ca^{2+}]_i$ was measured. No recordings were obtained during perifusion with solutions containing <6 mmol/l glucose. However, in other experiments, $[Ca^{2+}]_i$ was consistently low and stable in the presence of 4 mmol/l glucose (F.C.J., unpublished data). The typical response to higher glucose concentrations was characterized by repetitive transient elevations of $[Ca^{2+}]_i$ (Fig. 1). Sustained elevations of $[Ca^{2+}]_i$ were not observed, even at 20 mmol/l glucose. In some single cells, $[Ca^{2+}]_i$ oscillations were induced by 6 mmol/l glucose (cell 1), whereas other cells only responded to a higher glucose concentration (cell 2) or did not respond at all (cell 3). In glucose-sensitive cells, tolbutamide consistently increased the $[Ca^{2+}]_i$ rise produced by 20 mmol/l glucose. Most glucose-insensitive cells responded to tolbutamide (e.g., cell 3 in Fig. 1). In clusters, $[Ca^{2+}]_i$ responses were also

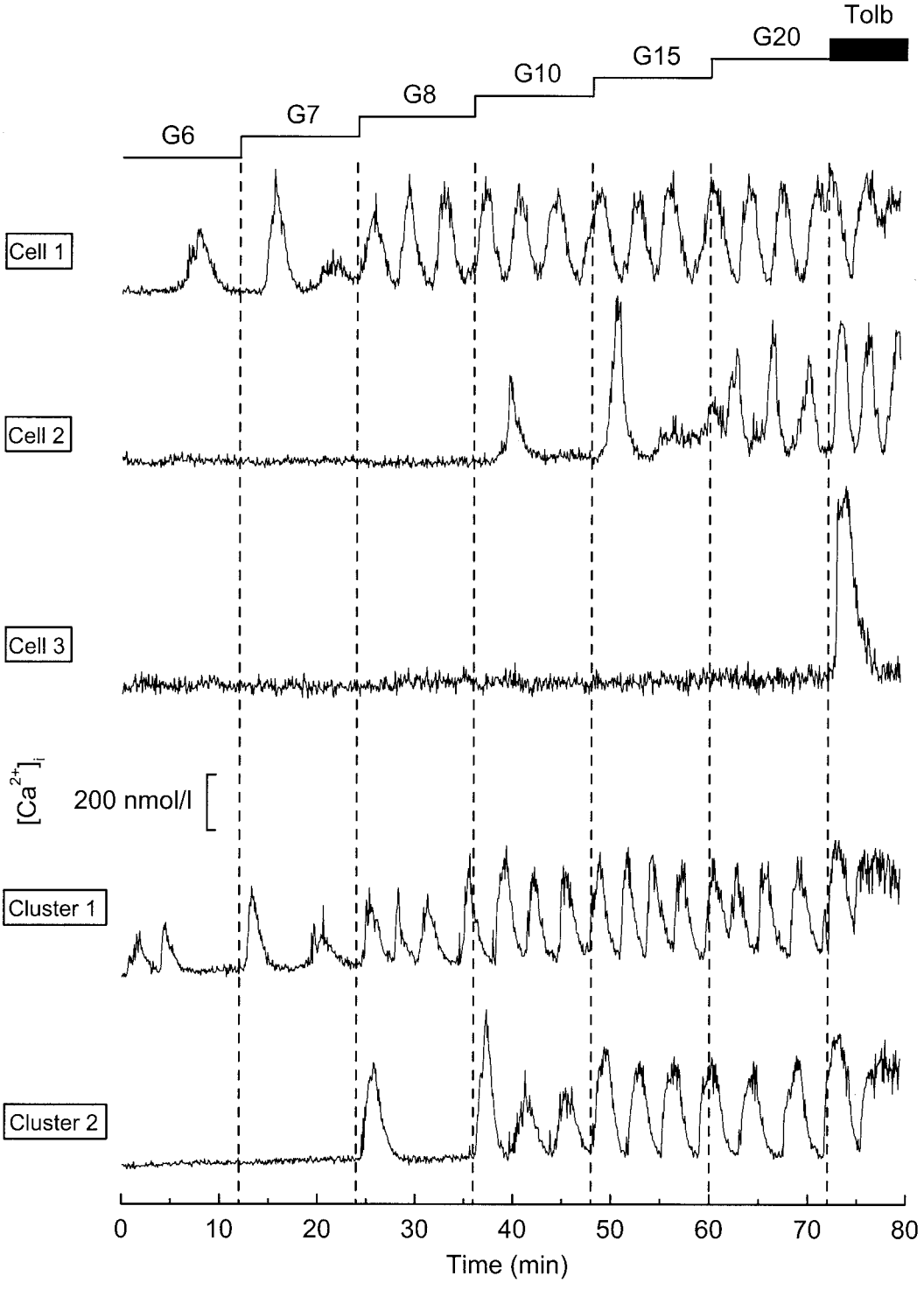

FIG. 1. Examples of $[Ca^{2+}]_i$ responses to increasing glucose (G) concentrations in islet single cells or clusters. The recording of $[Ca^{2+}]_i$ was started (time 0) after 10 min of perifusion with a medium containing 4 mmol/l glucose, simultaneously with the rise of the glucose concentration to 6 mmol/l. Each concentration of glucose was applied for 12 min. The experiment was terminated by addition of 100 μmol/l tolbutamide (Tolb) to the medium containing 20 mmol/l glucose. Cells 1 and 2 and clusters 1 and 2 showed different sensitivities to glucose. Cell 3 responded to tolbutamide but not to glucose. All preparations were cultured for 1 day. The quantification of these different responses is presented in Figs. 2 and 3.

characterized by large oscillations (no sustained elevation) but showed a lesser variability than in single cells. However, differences in the threshold concentration of glucose were also observed between clusters (Fig. 1). These differences were noted within the same preparation and did not simply reflect interpreparation variability.

The percentage of islet single cells showing a $[Ca^{2+}]_i$ rise in the presence of different glucose concentrations is shown in Fig. 2*A*. It was slightly less on day 2 than day 1, but the overall glucose dependency was not influenced by culture time. Whereas $[Ca^{2+}]_i$ was consistently low and stable in the presence of 4 mmol/l glucose (F.C.J., unpub-

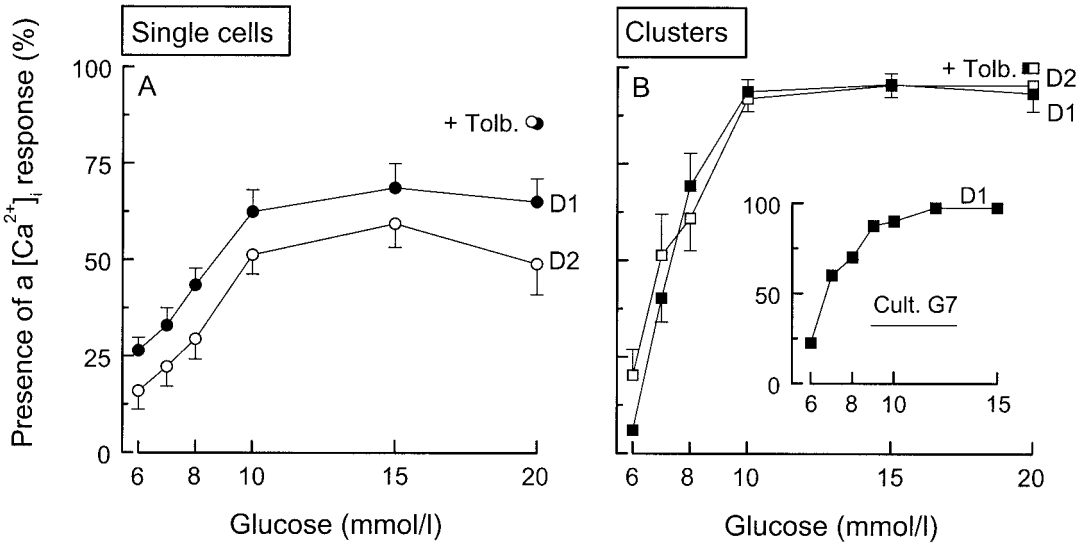

FIG. 2. Influence of the glucose concentration on the percentage of islet single cells (A) and clusters (B) showing a $[Ca^{2+}]_i$ response (recruitment). The preparations were cultured for 1 day (D1) or 2 days (D2) in the presence of 10 mmol/l glucose before being stimulated by increasing glucose concentrations and eventually by 100 μmol/l tolbutamide (Tolb.), as shown in Fig. 1. The total number of studied single cells were 190 (D1) and 190 (D2), and those of studied clusters were 47 (D1) and 53 (D2), from 10 different cultures. Values are means ± SE. The inset shows the results from an independent series of experiments including 40 clusters from seven cultures for 1 day (D1) in the presence of only 7 mmol/l glucose (Cult. G7).

lished data), ~20% islet single cells responded to 6 mmol/l glucose. The proportion increased with the glucose concentration and reached a plateau of ~60% between 10 and 15 mmol/l glucose. In the presence of tolbutamide, only 13% of the cells remained inert. None of these cells were dead according to the propidium iodide/acridine orange technique (see RESEARCH DESIGN AND METHODS); they were probably α-cells because these cells do not respond to tolbutamide in the mouse (33). The proportion of islet cell clusters showing a $[Ca^{2+}]_i$ response also increased between 6 and 10 mmol/l glucose and reached a maximum of 90–95% (Fig. 2*B*). The few clusters that were still inert in 20 mmol/l glucose alone all responded to tolbutamide. The results of this first series of experiments indicate that glucose recruits islet single cells and clusters to generate a $[Ca^{2+}]_i$ response.

We also measured the influence of the glucose concentration on the amplitude of the $[Ca^{2+}]_i$ response. In both single cells and clusters, glucose induced a concentration-dependent rise in mean $[Ca^{2+}]_i$ that reached a maximum at 15 mmol/l glucose (Fig. 3*A* and *B*). This rise was partly accounted for by the recruitment of active cells and clusters. However, when only those cells or clusters active at a given glucose concentration were taken into consideration, a rise in mean $[Ca^{2+}]_i$ was still observed as the glucose concentration was raised (Fig. 3*C* and *D*). The phenomenon can be seen in Fig. 1. In none of the 242 glucose-responsive cells and 95 glucose-responsive clusters did $[Ca^{2+}]_i$ suddenly switch from an oscillatory pattern to a sustained elevation. This indicates that glucose augments the amplitude of the individual response, and, as shown in Fig. 3*C* and *D*, this effect is larger in clusters than in single cells.

A second independent series of experiments was performed with clusters of islet cells cultured for 1 day in the presence of a lower concentration of glucose (7 instead of 10 mmol/l glucose). As shown by the insets in Figs. 2*B* and 3*D*, the recruitment of clusters and the rise in mean $[Ca^{2+}]_i$ in active clusters were similar to those observed in the first series. This indicates that our findings are not dependent on a specific duration of the culture or glucose concentration during the culture.

Influence of the glucose concentration on the $[Ca^{2+}]_i$ response in individual cells within clusters. The above data have shown that the glucose sensitivity of different clusters is variable but did not provide any information about the homogeneity of the response within each cluster. The third series of experiments, therefore, characterized the individual cell response in monolayer clusters. The upper trace in Fig. 4*A* illustrates the global $[Ca^{2+}]_i$ response in a cluster of 10 cells that started to respond in 6 mmol/l glucose. The four lower traces show that the signal was synchronous and of similar amplitude in individual cells. Figure 4*B* illustrates the response of another cluster (14 cells) that was also active at 6 mmol/l glucose. Although synchronous in all cells, the $[Ca^{2+}]_i$ response was of smaller amplitude in some cells (C3–C4) than in others (C1–C2). When this difference in amplitude was observed, it usually persisted even at higher glucose concentrations.

The characteristic synchronous $[Ca^{2+}]_i$ response to glucose is illustrated by the series of pseudocolor images of Fig. 5*A*, recorded in a cluster of eight cells. All cells showed simultaneous increases or decreases in $[Ca^{2+}]_i$. Cell recruitment within an active cluster was only rarely observed. In the cluster illustrated by Fig. 5*B*, one cell was active at 6 mmol/l glucose, whereas the other three cells remained silent. At 7 mmol/l glucose, a synchronized response occurred in the four cells, with sometimes a slightly greater amplitude in the first cell than in the others. $[Ca^{2+}]_i$ waves propagating across this or other clusters were not observed. In summary, one can consider that the progressive increase in the glucose concentration recruited clusters 4A, 4B, and 5B first, and then cluster 5A, but that no recruitment of individual cells occurred within

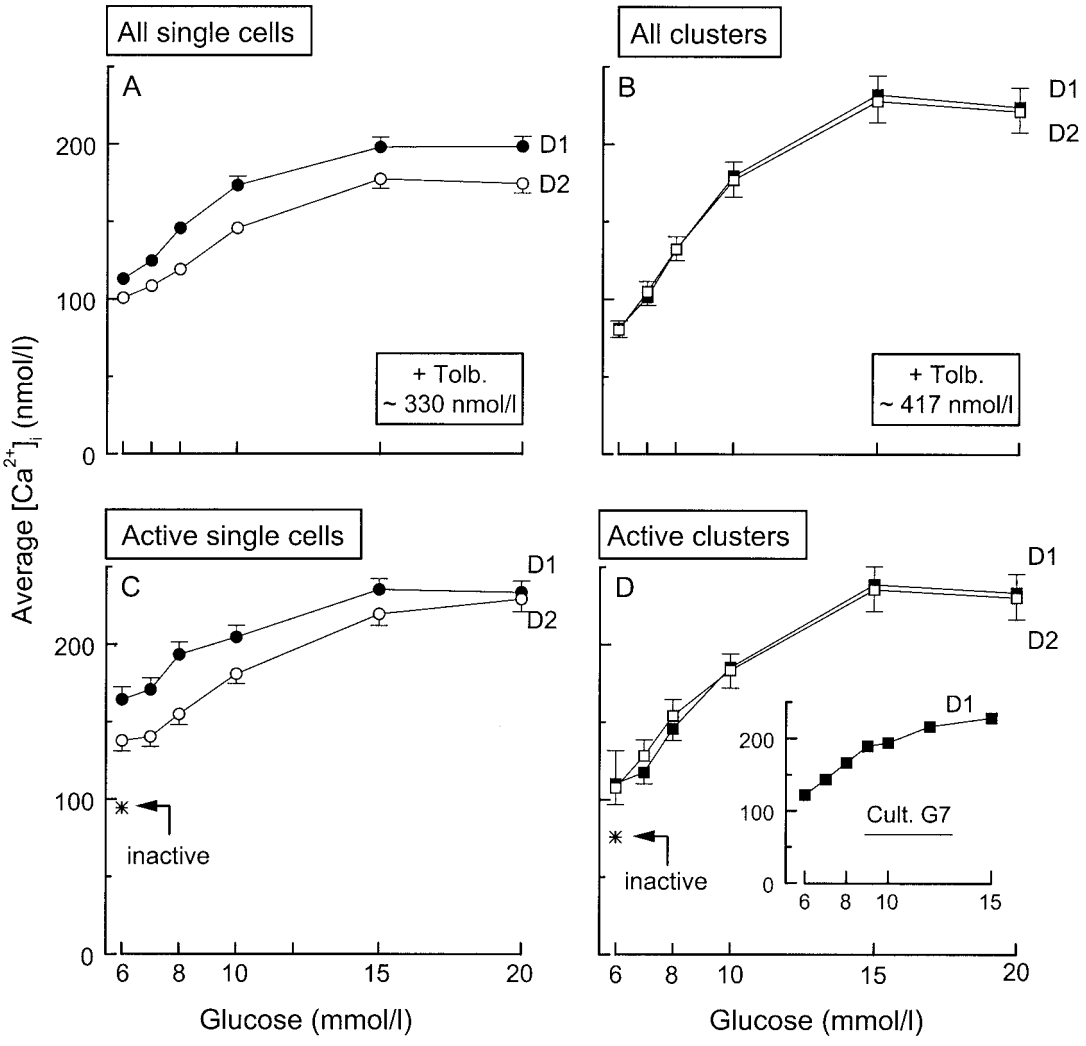

FIG. 3. Influence of the glucose concentration on average $[Ca^{2+}]_i$ in islet single cells and clusters. *A* and *B*: Average $[Ca^{2+}]_i$ was measured in all cells and clusters regardless of the presence or absence of a $[Ca^{2+}]_i$ response. The *n* values are thus identical at all glucose concentrations: 190 single cells 1 day (D1), 190 single cells 2 days (D2), 47 clusters D1, and 53 clusters D2. The observed increase in $[Ca^{2+}]_i$ therefore reflects both the increase in the proportion of responding cells and clusters and the increase in the individual responses. *C* and *D*: Only those cells and clusters showing a $[Ca^{2+}]_i$ response were included in the calculations. Hence, the *n* values are different at each glucose concentration. The curves therefore illustrate the increase in the individual responses. Values are means ± SE. The inset shows the increase in $[Ca^{2+}]_i$ measured in the clusters cultured for 1 day in the presence of only 7 mmol/l glucose (Cult. G7). The preparations used for these calculations were the same as those shown in Fig. 2*B*. Tolb., tolbutamide.

the active clusters, except in cluster 5B, in which one cell became active before the others.

The incidence of these different types of responses is presented in Fig. 6*A*. The whole columns show that the percentage of clusters with a $[Ca^{2+}]_i$ response increased with the glucose concentration to reach 100% at 10 mmol/l glucose. The black section of each column corresponds to those clusters in which not all cells were active. This proportion was small and decreased as the glucose concentration was raised (Fig. 6*A*). In other words, when a cluster responded to glucose by a $[Ca^{2+}]_i$ rise, the vast majority of cells or all cells contributed to the response. These observations indicate that individual cell recruitment within clusters is an infrequent phenomenon.

However, it remained possible that the synchronization of $[Ca^{2+}]_i$ oscillations was affected by glucose. The results of this analysis are shown in Fig. 6*B*. In the clusters that were active at 6-7 mmol/l glucose, $[Ca^{2+}]_i$ oscillations were synchronous in 85–90% of the cells. As the glucose concentration was raised, the regularity of the responses increased to characterize 95% of the clusters at 10 mmol/l glucose (Fig. 6*B*). The few nonresponsive or asynchronous cells within the active clusters may be non–β-cells (33).

Correlations between glucose-induced $[Ca^{2+}]_i$ responses and insulin secretion. In a fourth series of experiments, we directly compared the effects of glucose on cytosolic $[Ca^{2+}]_i$ and insulin secretion in the same preparations of islet single cells and clusters. Figure 7*A* shows the mean changes induced by six glucose concentrations tested in sequence. The $[Ca^{2+}]_i$ trace corresponds to the average changes in all single cells and clusters present in the observation field, and the insulin secretion profile reflects the activity of all cells attached to the coverslip. Raising the glucose concentration from 4 to 7 mmol/l caused a large peak followed by a smaller sustained elevation of both $[Ca^{2+}]_i$ and insulin secretion. Subsequent increases in the glucose concentration induced progressive parallel elevations of $[Ca^{2+}]_i$ and insulin secretion. The average integrated increases in $[Ca^{2+}]_i$ and insulin secretion above basal levels are shown in Fig. 7*B*

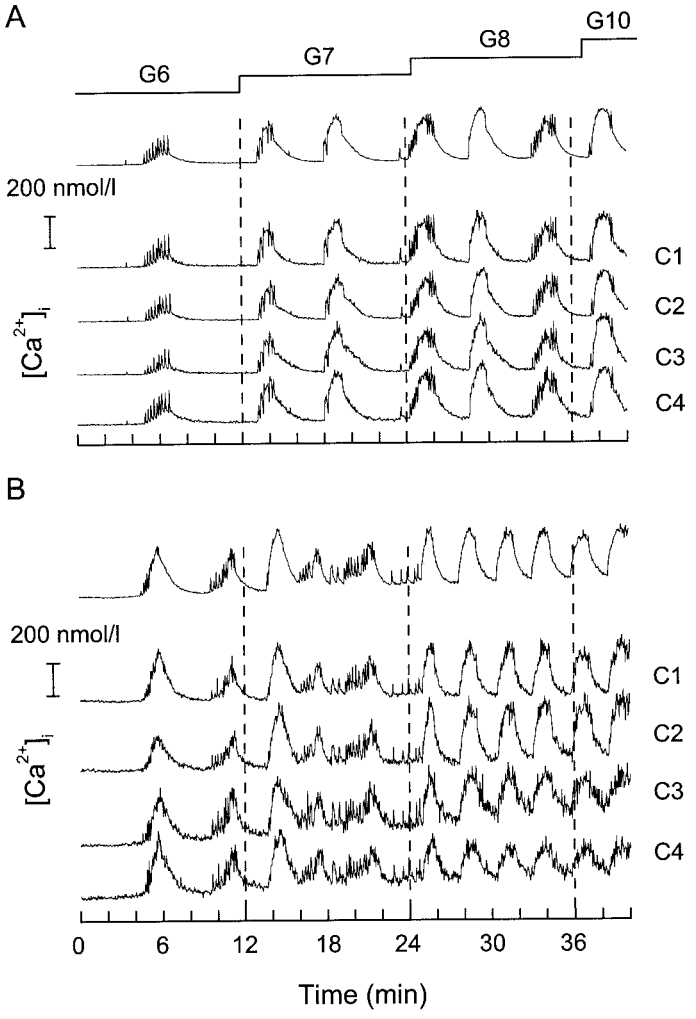

FIG. 4. Examples of the homogeneity of the $[Ca^{2+}]_i$ responses to increasing glucose (G) concentrations in islet cell clusters. Clusters forming monolayers were selected to permit analysis of $[Ca^{2+}]_i$ in individual cells without the confounding problem of active cells masking inactive ones in another layer. Successive $[Ca^{2+}]_i$ measurements were obtained at 2.4-s intervals. The upper trace shows the global response of the cluster and the lower traces show the response of four individual cells (C1–C4). The changes in $[Ca^{2+}]_i$ were synchronous in all cells of both clusters, but their amplitude was either similar (A) or variable (*B*) between cells of the cluster.

and *C*. Both parameters displayed a similar glucose dependency.

Finally, the glucose dependency of insulin secretion was compared with that of the recruitment of islet single cells and clusters from the same preparations (Fig. 8). A $[Ca^{2+}]_i$ response was induced in the majority of clusters and responsive cells already by 7 mmol/l glucose, whereas insulin secretion kept increasing up to 15–20 mmol/l glucose. Glucose-induced insulin secretion, therefore, cannot entirely be ascribed to the recruitment of β-cells to generate a $[Ca^{2+}]_i$ signal. The increase in the individual cell response (Fig. 7) certainly plays a major role.

DISCUSSION

Isolated single β-cells display heterogeneous metabolic, biosynthetic, and secretory responses to glucose. Because of their different threshold sensitivities to glucose, increasing numbers of cells become active (are recruited) as the sugar concentration is raised (10). It is also widely accepted that single β-cells exhibit heterogeneous $[Ca^{2+}]_i$ responses to glucose (34–38). However, only one aspect of this heterogeneity is well established: the pattern of the response to a stimulatory concentration of glucose is irregular and variable. In contrast, only limited evidence, based on the use of few glucose concentrations, suggests the existence of a variable sensitivity to glucose (35,37).

The present study clearly establishes that the threshold glucose concentration inducing a $[Ca^{2+}]_i$ rise is variable between individual isolated mouse β-cells. The proportion of β-cells showing an elevation of $[Ca^{2+}]_i$ increases with the rise in glucose concentration. The phenomenon of recruitment thus also exists at the $[Ca^{2+}]_i$ level. Interestingly, the proportion of 60–65% active cells in 15 mmol/l glucose is in agreement with the percentage of rat β-cells secreting insulin (as shown by reverse hemolytic plaque assay) in response to a similar glucose concentration (14, 15,39). This similarity suggests that, when glucose recognition (metabolism and subsequent steps) is sufficient to lead to a $[Ca^{2+}]_i$ rise, it also leads to insulin secretion in individual cells.

The major aim of our study, however, was not to characterize glucose-induced $[Ca^{2+}]_i$ changes in isolated cells but to assess whether recruitment also exists in a more physiological situation, when β-cells are associated in clusters that may be more representative of their situation within islets. The results show that the threshold glucose concentration inducing a $[Ca^{2+}]_i$ response is also variable between clusters. Raising the glucose concentration recruited more and more active clusters. The difference with single cells did not reside in the glucose sensitivity (K_m between 7 and 8 mmol/l for both types of preparations) but in the maximum response. All or practically all clusters responded to glucose compared with 60–65% of single cells. The inclusion of unrecognized α-cells in the studied single cells probably contributes to but cannot entirely explain the difference.

Whereas some heterogeneity was observed between clusters, the individual cell response within clusters was more homogeneous. No more than 25% of the clusters responding to 6 mmol/l glucose included unresponsive cells. This proportion decreased close to zero as the glucose concentration was raised to 8–10 mmol/l. Moreover, the synchrony of the response was the rule; asynchronous $[Ca^{2+}]_i$ changes in neighboring cells occurred in no more than 15% of the active clusters at 6-7 mmol/l glucose, and this proportion decreased with the rise in glucose. Recruitment of individual cells within clusters rarely occurs; when a cluster is recruited, all or nearly all its cells respond. $[Ca^{2+}]_i$ measurements by confocal microscopy in intact mouse islets have shown that α- and δ-cells (subsequently identified by immunocytochemistry) display distinct responses from those of β-cells (33,40). It is thus possible that the few nonresponsive or asynchronous cells within clusters are non–β-cells. On the other hand, because α-cells can be coupled with β-cells in vitro (41), we cannot exclude the possibility that some non–β-cells are entrained by β-cells within clusters.

Our results further show that average $[Ca^{2+}]_i$ in single cells and clusters increases with the glucose concentration. This increase corresponds to both the recruitment of active cells and a change in the magnitude of the individual

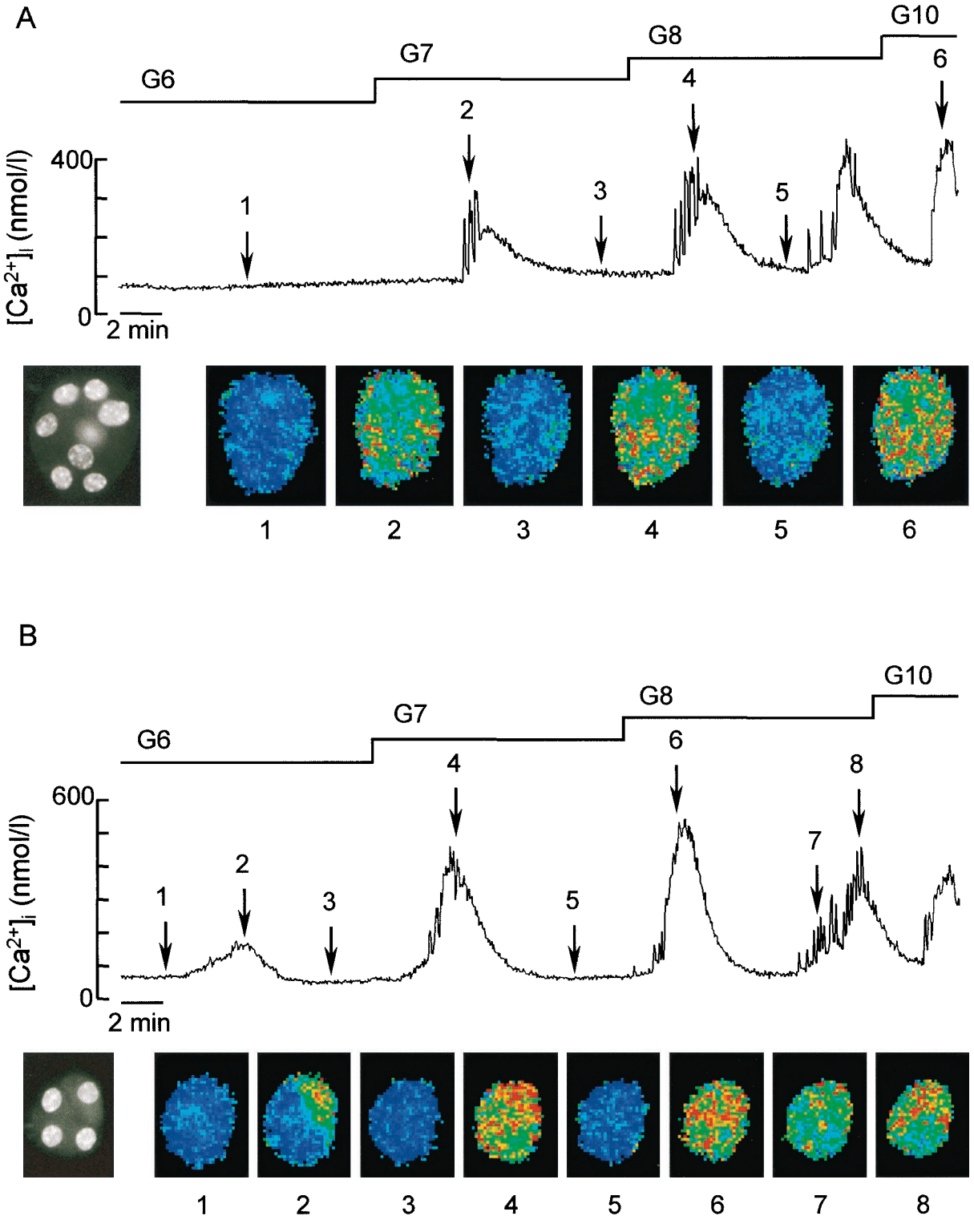

FIG. 5. Illustration of the homogeneity or heterogeneity of the $[Ca^{2+}]_i$ responses to increasing glucose (G) concentrations in islet cell clusters. The left-hand pictures show the analyzed clusters after staining of the nuclei with bisbenzimide. The series of color pictures show pseudocolor images of $[Ca^{2+}]_i$ in the clusters at the time indicated by arrows (blue corresponds to low $[Ca^{2+}]_i$ and red to high $[Ca^{2+}]_i$). The traces show the integrated $[Ca^{2+}]_i$ changes with time. *A*: Cluster of eight cells, with one nucleus slightly out of focus; all cells responded synchronously. *B*: Cluster of four cells; the upper right cell only responded to 6 mmol/l glucose; subsequently all four cells responded synchronously.

cell response. In previous studies using cultured β-cells from *ob/ob* mice (36,37), the glucose-dependent increase in $[Ca^{2+}]_i$ was ascribed to recruitment of individual cells showing abrupt transitions, at variable glucose concentrations, between three states: low basal $[Ca^{2+}]_i$, oscillatory $[Ca^{2+}]_i$, and steadily elevated $[Ca^{2+}]_i$ (reached by 17 and 40% of the cells in 11 and 20 mmol/l glucose, respectively) (36). Such abrupt changes between oscillations and sustained elevations of $[Ca^{2+}]_i$ were never observed in our preparations, in which the glucose-dependent increase in

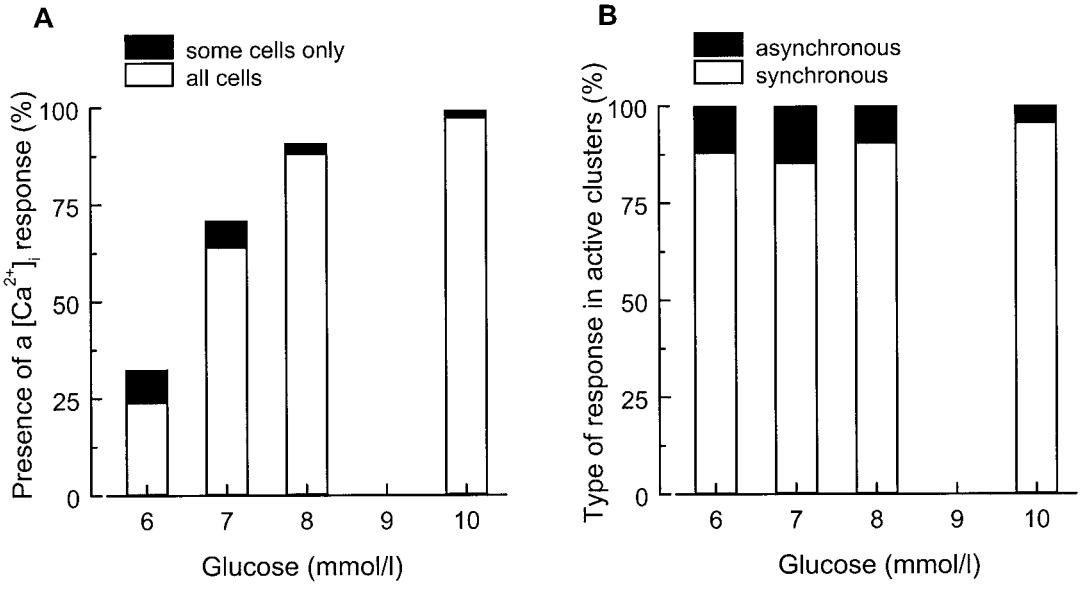

FIG. 6. Homogeneity of the $[Ca^{2+}]_i$ responses induced by increasing glucose concentrations in islet cell clusters. Preparations cultured for 1 or 2 days were stimulated by increasing glucose concentrations as shown in Fig. 4. Because the distribution of the different groups was similar after 1 and 2 days, the data were pooled. *A*: Percentage of clusters in which the $[Ca^{2+}]_i$ response was present in all cells or in some cells only. The total number of studied clusters was 120. *B*: Percentage of the active clusters in which the $[Ca^{2+}]_i$ response was synchronous or asynchronous. The sum of the two columns is thus 100%, but the number of clusters was different at each glucose concentration.

$[Ca^{2+}]_i$ was more gradual. There is no doubt that the $[Ca^{2+}]_i$ response of individual β-cells to glucose is not of an all-or-none type. Finally, we did not observe $[Ca^{2+}]_i$ waves propagating across the clusters. This is in contrast with a previous study (42) that described such propagations in clusters of β-cells from *ob/ob* mice tested 3 h after dispersion of the islets; two examples were shown, but the incidence of the phenomenon was not given. These waves were attributed to electrical coupling of the cells. In another study, glucose-induced $[Ca^{2+}]_i$ waves propagating within monolayers of cultured β-cells and also between physically separated clusters have been ascribed to rhythmic release and diffusion of unknown stimulating factors (43). We cannot exclude the possibility that we have missed $[Ca^{2+}]_i$ waves propagating too fast for the resolution of our system or propagating only over distances exceeding the size of the studied clusters.

An important observation of the present study is that the recruitment of β-cells occurs over a narrow range of glucose concentrations: 6–10 mmol/l. Our findings are in complete agreement with the glucose dependency of the appearance of electrical activity in β-cells within intact mouse islets (23–25). This electrical activity indeed reflects Ca^{2+} influx, the major mechanism underlying the glucose-induced $[Ca^{2+}]_i$ rise (2–4,29). By synchronizing the changes in membrane potential, electrical coupling (44,45) synchronizes Ca^{2+} influx and thereby minimizes the heterogeneity of the triggering signal of β-cells in situ. However, the quantitative correlation is not perfect. As already pointed out by others (36), Ca^{2+}-dependent electrical activity in intact islets is more finely regulated by the changes in glucose concentration than are the $[Ca^{2+}]_i$ oscillations in single β-cells or clusters.

We do not believe that paracrine effects explain the high incidence and good synchronization of the $[Ca^{2+}]_i$ responses in clusters. Thus, no more than 42% of the clusters contained at least one non–β-cell, whereas over 95% of the clusters stimulated by 10 mmol/l glucose displayed a synchronous response. Moreover, isolated non–β-cells were scattered among single cells and clusters. There is thus no reason why the small amounts of hormone that they release (little glucagon is expected to be secreted under our experimental conditions) should differentially influence isolated β-cells and clusters of β-cells in a constantly perifused system. It has also been suggested that oscillations of the K^+ concentration in the confined extracellular space of the islet might contribute to the synchronization of the membrane potential changes in β-cells (46). Such a mechanism is unlikely to remain operative in our model of perifused monolayer clusters.

Recruitment of β-cells into an active secretory state by increases in the glucose concentration has been described in preparations of isolated single β-cells maintained in culture (11–15) or tested several hours after islet isolation (47). Whether the phenomenon exists under physiological conditions, when β-cells are associated within islets, has not been established because secreting and nonsecreting cells cannot be readily distinguished. The problem was approached by recording the triggering signal of glucose-induced insulin secretion—the rise in $[Ca^{2+}]_i$. In fresh and cultured mouse islets, half-maximum and maximum stimulation of insulin secretion are produced by ~15 and 30 mmol/l glucose, respectively (48). The concentration dependency of insulin secretion by our preparations of mouse islet cells and clusters was clearly shifted to the left. We have no explanation for this difference, which does not seem to have been reported (and studied) previously. Nevertheless, the recruitment of β-cells to induce a $[Ca^{2+}]_i$ response was even more sensitive to glucose, which indicates that the phenomenon only partially accounts for glucose-induced insulin secretion.

This conclusion is based on the evidence that, except under artificial conditions of combined and strong activation of protein kinase A and protein kinase C (9,49),

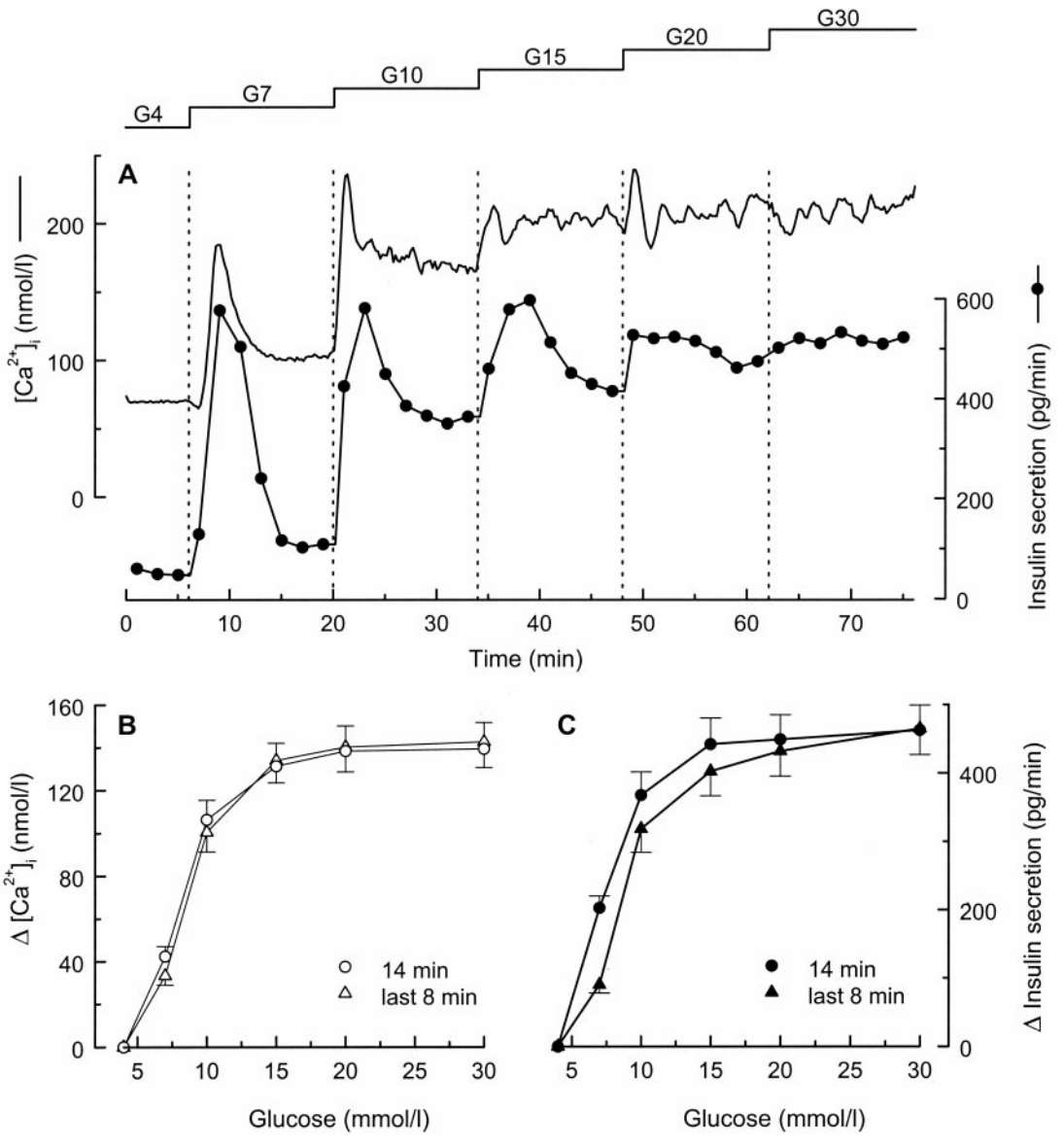

FIG. 7. Simultaneous measurements of $[Ca^{2+}]_i$ and insulin secretion in preparations of islet single cells and clusters. *A*: Mean changes of $[Ca^{2+}]_i$ and insulin secretion in 24 experiments with preparations from 12 cultures. Peaks and nadirs of $[Ca^{2+}]_i$ oscillations are erased by averaging. The glucose (G) concentration was increased stepwise as indicated, from 4 to 30 mmol/l (G4–G30). *B*: Increase of $[Ca^{2+}]_i$ above basal concentration at 4 mmol/l: mean $[Ca^{2+}]_i$ was calculated for the 14 min or only last 8 min of stimulation with each glucose concentration. *C*: Increase of insulin secretion above the basal rate at 4 mmol/l glucose: mean secretion rate was calculated for each period of stimulation (14 min or only last 8 min). Values are means ± SE for 24 experiments.

glucose does not increase insulin secretion if $[Ca^{2+}]_i$ does not rise in β-cells or is not already elevated above basal levels (1). However, the reverse is not necessarily true. There is no proof that all β-cells displaying a $[Ca^{2+}]_i$ rise in a glucose-stimulated islet or cluster secrete insulin. Three theoretical possibilities can be envisaged. First, the $[Ca^{2+}]_i$ signal is present in all cells, but its magnitude is different. It is not exceptional to observe β-cells within a cluster (e.g., Fig. 4*B*) or groups of β-cells within whole islets (29), in which $[Ca^{2+}]_i$ is elevated to a lesser extent than elsewhere in the preparation. This difference usually persists throughout the whole range of glucose concentrations and its significance is unclear. Second, glucose metabolism may be similar in all β-cells, as suggested by cell-sized NAD(P)H measurements in intact islets (19), but the efficacy of a similar $[Ca^{2+}]_i$ signal on secretion may be modulated by paracrine influences. However, it is not immediately apparent which paracrine signal could positively affect increasing numbers of β-cells as the glucose concentration is raised. Third, subtle metabolic differences exist between β-cells in situ, which either are beyond the resolution of NAD(P)H measurements or involve signals undetected by this method. In this case, the action of Ca^{2+} on exocytosis could be different; in other words, a similar triggering $[Ca^{2+}]_i$ signal could lead to different secretory responses because of differences in the amplifying action of glucose (1,5–8).

In conclusion, β-cell recruitment by glucose can occur at the stage of the $[Ca^{2+}]_i$ response. However, this type of recruitment is restricted to a narrow range of glucose concentrations, particularly when β-cell association decreases the heterogeneity of the responses. Recruitment of

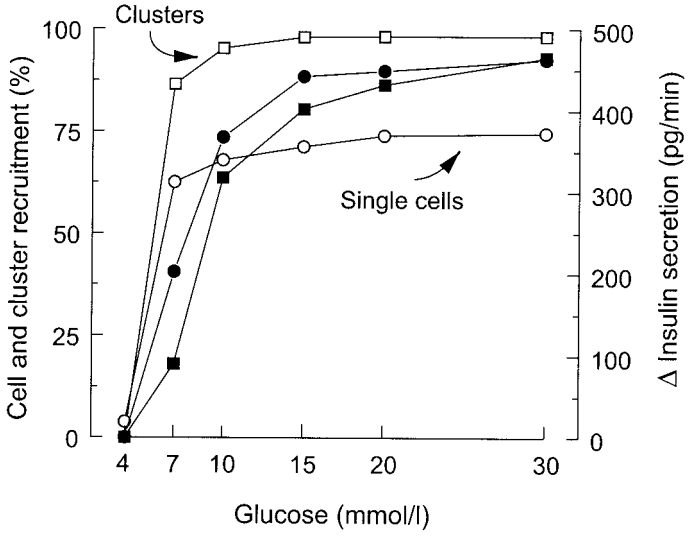

FIG. 8. Influence of the glucose concentration on the appearance of a $[Ca^{2+}]_i$ response (recruitment) in islet single cells (○) and clusters (□) and on insulin secretion for the 14 min (●) or only the last 8 min of stimulation (■). All data were obtained from the same preparations as those shown in Fig. 6. Note that the vast majority of β-cells (at least 85%) are situated within clusters, which, therefore, are the major contributors to insulin secretion.

β-cells to generate a $[Ca^{2+}]_i$ response contributes to, but does not entirely explain, glucose-induced insulin secretion. The increase in $[Ca^{2+}]_i$ in individual β-cells plays a major role. If heterogeneity of insulin secretion exists in situ, it probably occurs at a step of stimulus secretion coupling downstream of the $[Ca^{2+}]_i$ rise. This step could be a modulation of the action of Ca^{2+} on exocytosis through the amplifying effect of glucose (1).

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