# Identification of α- and β-Cells in Intact Isolated Islets of Langerhans by Their Characteristic Cytoplasmic $Ca^{2+}$ Concentration Dynamics and Immunocytochemical Staining

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**Ratiometric images of cytoplasmic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$) in individual cells were recorded simultaneously with a confocal ultraviolet-laser microscope in the Indo-1-loaded islets isolated from mice. After changes in $[Ca^{2+}]_c$ in response to glucose or amino acids were recorded, the islet was fixed, permeabilized, and stained by the indirect immunofluorescence method against insulin or glucagon in situ; the individual cells were then identified in the focal plain identical to that used for the $[Ca^{2+}]_c$ imaging. Almost all cells identified as insulin-positive (β-cells) by their distinct immunofluorescence responded to the increase in glucose concentration from 3 to 11 mmol/l with an increase in $[Ca^{2+}]_c$. Major populations of cells (~65%) identified as glucagon-positive (α-cells) responded to the addition of arginine (5–10 mmol/l) to 3 mmol/l glucose solution with an increase in $[Ca^{2+}]_c$. About half of the α-cells (47.6%) responded to the addition of alanine (5–10 mmol/l) to 3 mmol/l glucose solution with an increase in $[Ca^{2+}]_c$. In contrast, <13% of β-cells responded to the addition of alanine (5–10 mmol/l) or arginine (5–10 mmol/l) to 3 mol/l glucose with an increase in $[Ca^{2+}]_c$. More than one-fourth of α-cells responded with an increase in $[Ca^{2+}]_c$ when glucose concentration in perifusion solution was reduced from 11 to 0 mmol/l. These results indicate that $[Ca^{2+}]_c$ changes in islet cells stimulated by glucose or amino acid were characteristic of the cell species, at least in the α- and β-cell. This technique provides a useful tool to investigate not only the intracellular signal transduction but also the intercellular signal transmission in the intact islet. *Diabetes* 47:751–757, 1998**

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$[Ca^{2+}]_c$, cytoplasmic $Ca^{2+}$ concentration; FITC, fluorescein isothiocyanate; HK, HEPES-buffered Krebs solution; PBS, phosphate-buffered saline; UV, ultraviolet.

Pancreatic islets are composed of a community of four kinds of endocrine cells: α-, β-, δ-, and pancreatic polypeptide (PP) cells (1–3). Secretion of individual hormones from β-cells and α-cells in the pancreatic islet is regulated by concentrations of glucose and other nutrients in the extracellular environment: an increase in glucose level induces insulin release from β-cells, and a decrease of glucose causes glucagon release from α-cells. In the stimulus-secretion coupling of α- and β-cells, cytoplasmic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$) is known to play a cardinal role. In the β-cell in the islets, glucose transported into the cell increases ATP concentration, which leads to closure of $K^+$ channels and depolarization of the membrane potential. Then $Ca^{2+}$ enters from the extracellular space through voltage-dependent $Ca^{2+}$ channels (4–8). Finally, the resultant increase in $[Ca^{2+}]_c$ evokes insulin release. A characteristic feature of the $[Ca^{2+}]_c$ response to glucose in β-cells has been studied using dispersed single cell preparations (9–11), cluster cells (12), or isolated whole pancreatic islet preparations (13–16) by the image analysis with a conventional fluorescence microscope or by the microfluorometry with fura-2. The glucose-induced increase in $[Ca^{2+}]_c$ in the whole islet has been regarded as an intracellular signal in β-cells, because β-cells are major constituents in the islet. But, possible changes in $[Ca^{2+}]_c$ in response to glucose or other nutrients in minor constituents of the islets, non–β-cells, can hardly be distinguished in the ratiometric images obtained by conventional fluorescent microscopy. It is highly possible that the $[Ca^{2+}]_c$ dynamics in non–β-cells are masked by the major $[Ca^{2+}]_c$ response in β-cells. In fact, $[Ca^{2+}]_c$ responses in non–β-cells were demonstrated only in dispersed single cells (17–19) or single cells of cloned cell line (20). Moreover, in such preparations, intercellular communication between the islet cells was either absent or lost and, therefore, it has not been possible to study responses of intact islet cells to various physiological stimuli.

In the present study, we report discrete $[Ca^{2+}]_c$ responses to glucose and amino acids in individual α- and β-cells in an intact islet using confocal ultraviolet (UV)-laser scanning microscopy (21,22). Immunocytochemical identification of peptide hormones contained in individual cells was subsequently performed on the identical confocal plain of the whole intact islets.

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## RESEARCH DESIGN AND METHODS

**Isolation and primary culture of islets.** After cervical dislocation followed by exsanguination, the islets were isolated from male ICR mice (35–45 g body wt) by collagenase (type IV; Worthington, Freehold, NJ) digestion as previously described (23). Once isolated, islets were cultured for 1–3 days in RPMI 1640 medium (containing 11.1 mmol/l glucose, 10% fetal calf serum, 100 µg/ml streptomycin, and 100 IU/ml penicillin) at 37°C with an atmosphere of 95% air and 5% $CO_2$.

The standard solution used for the isolation of islets and for the $[Ca^{2+}]_c$ imaging experiment was a HEPES (4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid)-buffered Krebs solution (HK) containing (in mmol/l) 130 NaCl, 5 KCl, 5 $NaHCO_3$, 1 $NaH_2PO_4$, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 3 glucose. It was gassed with $O_2$ and supplemented with 1 mg/ml bovine serum albumin (fraction V; Sigma, St. Louis, MO). Its pH was adjusted to 7.4 with NaOH. Isolated and cultured islets were stimulated by adding glucose, arginine (HCl salt), or alanine to the perifusion solution.

**Indo-1 loading and confocal imaging of $[Ca^{2+}]_c$ in the islet cells.** An aliquot (80 µl) of HK containing 10 µmol/l Indo-1 acetoxymethyl ester (Indo-1/AM; Dojindo Laboratories, Kumamoto, Japan) and 0.025% cremophor EL was poured into a handmade perifusion chamber (5 mm diameter × 5 mm depth) equipped with a Cell-Tak (Collaborative Research, Bedford, MA) coated coverslip bottom. Cultured islets were washed with HK, and transferred to the chamber, and loaded with Indo-1/AM (final concentration, 8.3 µmol/l) for 60 min at a room temperature (~23°C). Then the chamber was placed on the stage of an inverted microscope (TMD-300; Nikon, Tokyo, Japan), and the islets were perifused with HK at a flow rate of 1 ml/min using a peristaltic pump. The temperature of the solution in the chamber was kept at 34°C or 26°C by warming the inlet tube of the perifusion solution. Confocal images of $[Ca^{2+}]_c$ were acquired by an UV-laser confocal image analysis system (RCM 8000, Nikon), as previously described in detail (22). In brief, islets were irradiated through a 40 × water-immersion objective (NCF Fluor N. A. 1.15, Nikon) with an excitation UV beam (351 nm) produced by an argon ion laser tube at a scanning frequency of 30 Hz. A focal plain was set <20 µm from the bottom of an islet, so that cells located on the surface region of the islet were tested here. Fluorescence emitted by the islets was guided to a pinhole diaphragm, separated at 440 nm with a dichroic mirror. The fluorescence intensities at two wavelengths (>440 nm and <440 nm) were detected with two photomultipliers. In actual $[Ca^{2+}]_c$ imaging experiments, an islet was irradiated for 8/33 s at every 10 s; eight fluorescence images were acquired and integrated; and then a pair of resultant images at each wavelength was stored into an optical disk as raw data.

The excitation laser power was lowered and a larger pinhole was selected to enable recording of Ca-images over 30 min, because Indo-1 showed rapid bleaching and cells received a photodynamic damage when they were continuously exposed to a laser beam of a high power. The optical thickness of Indo-1 ratio images was set at no less than 3 µm, and as a result, the sharpness of the image was relatively low compared with that of the immunostained fluorescence image. Each image consisted of 512 × 484 pixels, and each pixel had a spatial resolution of ~0.3 µm. The ratios of each pair of images (<440/>440) were calculated pixel by pixel to produce ratio images, and the ratio images were represented by a pseudocolor. The $[Ca^{2+}]_c$ was estimated from the ratio ($R$) using the following (24)

$$[Ca^{2+}]_c = K_d \times (R - R_{min})/(R_{max} - R) \times (F_0/F_S),$$

where $K_d$ is the dissociation constant for Indo-1/$Ca^{2+}$ complex (250 nmol/l), $R_{min}$ and $R_{max}$ are the ratios for bound and unbound forms for the Indo-1/$Ca^{2+}$ complex, respectively, and $F_0/F_S$ is the ratio of the fluorescence intensity at >440 nm at minimum and saturated $Ca^{2+}$ concentrations. $R_{min}$, $R_{max}$, and $F_0/F_S$ were acquired using ready-made $Ca^{2+}$-EGTA buffer solutions (Molecular Probes, Eugene, OR) in vitro.

**Immunocytochemical identification of islet cells.** α-Cells or β-cells in the islets used for the $[Ca^{2+}]_c$ imaging experiments were identified using indirect immunofluorescence staining (25) for insulin or glucagon. After the $[Ca^{2+}]_c$ responses were measured, the islets were fixed by replacing HK in the chamber with 10 mmol/l phosphate-buffered saline (PBS) containing 1% paraformaldehyde. After the fixation for 3 h at a room temperature, the islets were permeabilized with repetitive perifusion with PBS containing 0.3% Triton X-100 (PBS/T) for 1 h at a room temperature. Then the solution was replaced with a guinea pig anti-porcine insulin serum (Dakopatts, Denmark) diluted in 1:800 or with a rabbit anti-porcine glucagon serum (Dakopatts) diluted in 1:300; the islets were incubated overnight at 4°C. The islets were washed with perifusion with the PBS/T for 30 min and incubated in a fluorescein isothiocyanate (FITC)-conjugated goat anti-guinea pig IgG serum (for insulin; Dakopatts) diluted in 1:80 or in a FITC-conjugated goat anti-rabbit IgG serum (for glucagon; donated by Dr. S. Ito, Niigata University) diluted in 1:80 for 1.5 h at a room temperature. After the islets were washed with the PBS/T for 30 min, confocal images of FITC-labeled islets were acquired with the same system used for the $[Ca^{2+}]_c$ imaging. The identical islet was irradiated with an excitation laser beam (488 nm) at a scanning frequency of 30 Hz, and fluorescence intensity (>545 nm) was detected with a photomultiplier. A confocal image of the FITC-labeled islets (512 × 484 pixels, spatial resolution; ~0.3 µm) was produced by integration of 64 fluorescence images.

## RESULTS

**Glucose-induced $[Ca^{2+}]_c$ response in β- and α-cells in intact islets.** Indo-1–loaded mouse pancreatic islets were perifused with HK for about 15 min and were examined under a phase contrast microscope before $[Ca^{2+}]_c$ imaging. Individual cells in intact mouse islets were distinguishable in an Indo-1 ratio image, because $[Ca^{2+}]_c$ was slightly different in individual cells. The mean value of basal $[Ca^{2+}]_c$ of islet cells before any stimulation was 116.8 nmol/l, calculated from 230 cells, and the fluctuations of $[Ca^{2+}]_c$ in individual cells at rest were in the range of ±8.9 nmol/l. The $[Ca^{2+}]_c$ response of the islet cells to a change in glucose concentration or to addition of amino acids was judged significant when $[Ca^{2+}]_c$ increased by >50 nmol/l after the treatments.

Figure 1 shows the islet labeled with the specific insulin antibody after $[Ca^{2+}]_c$ imaging. The islets were fixed and stained in situ in the perfusion chamber, and both images were acquired by the same microscopy with the same optical setting, so that immunocytochemically stained images of the islet were acquired at the identical focal plain of $[Ca^{2+}]_c$ images. $[Ca^{2+}]_c$ responses in β-cells and those in α-cells could be differentiated from each other within the intact islet. Fluorescence intensities of the cytoplasmic region of islet cells showed two peaks in the histogram (Fig. 1*B*) and could be separated into two groups. In Fig. 1*B*, cells with >100 units in mean intensity were stained uniformly in cytoplasmic area and were judged as insulin-positive cells (β-cells). In this islet, 17 of 28 cells were found to be insulin-positive. When the islet was perifused with the solution containing 5 mmol/l alanine with 3 mmol/l glucose at 34°C, β-cells did not respond to 5 mmol/l alanine (cells b and c in Fig. 1, for example). The same β-cells responded to an increase in glucose to 11 mmol/l by exhibiting a recurrent rise in $[Ca^{2+}]_c$ (Fig. 1*A* and *C*). The $[Ca^{2+}]_c$ of β-cells rose rapidly, reached >500 nmol/l, and then returned to the prestimulation level in a few minutes. The temporal sequences of the $[Ca^{2+}]_c$ rise in individual β-cells were synchronized in the same islet. In contrast, none of the insulin-negative cells responded to an increase in glucose concentration. In total, 91.5% of insulin-positive cells (227/248 cells) and 26.1% of insulin-negative cells (17/69 cells) were responsive to the increase in glucose concentrations (Fig. 2*A*), but the responses in the insulin-negative cells were much slower in the rise time and had smaller amplitudes than did those in the insulin-positive cells (Table 1).

When another islet was stimulated with 11 mmol/l glucose, two of seven glucagon-positive cells in the islet revealed a weak (slow in rise and a small amplitude) and monophasic $[Ca^{2+}]_c$ rise in response to glucose (cell b in Fig. 3 and Table 1). After the response, six of seven α-cells in the islet responded to the deprivation of glucose in the solution by exhibiting recurrent rise in $[Ca^{2+}]_c$ (Fig. 3*A* and *C*). In α-cells (cells a, b, and e in Fig. 3), $[Ca^{2+}]_c$ reached a high level, which was comparable to the peak in $[Ca^{2+}]_c$ observed in glucagon-negative cells during glucose stimulation, after removal of glucose from the perifusion solution ("glucose-off response"). In total, 26.7% of the α-cells (8/30 cells) revealed such glucose-off responses (Fig. 2). On the contrary, 15 of 19 glucagon-negative cells in the islet responded to the increase in glucose concentration with

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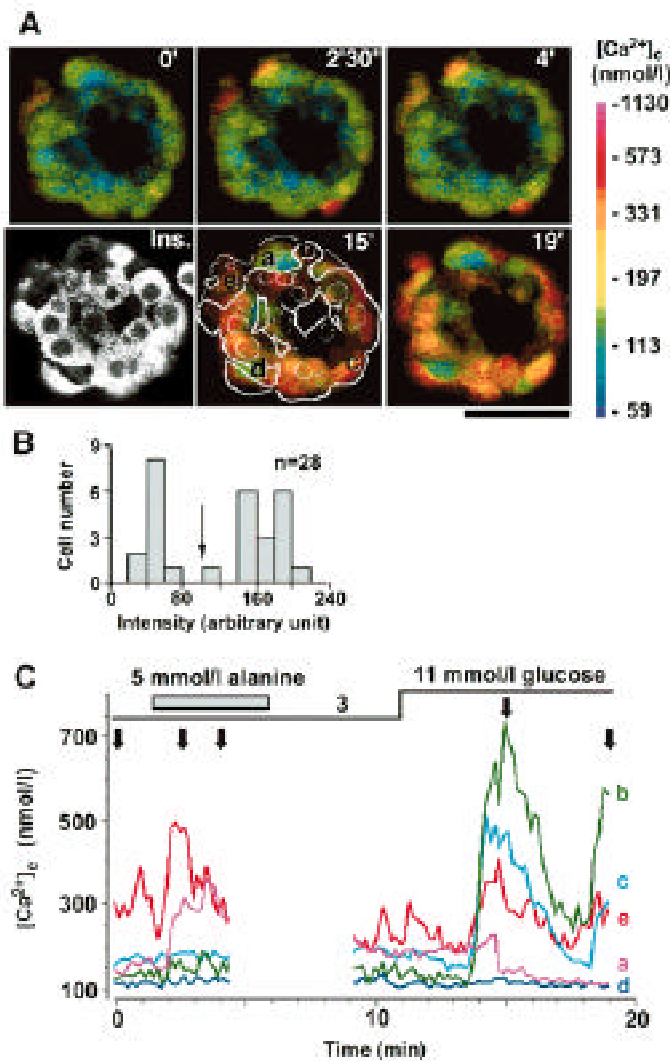


FIG. 1. $[Ca^{2+}]_c$ responses and insulin immunofluorescence in an intact islet perifused with 5 mmol/l alanine followed by 11 mmol/l glucose at 34°C. *A:* Pseudocolor images of the spatial and temporal $[Ca^{2+}]_c$ changes before and during perifusion with 3 mmol/l glucose stimulation containing 5 mmol/l alanine and with 11 mmol/l glucose solution. Images were obtained by ratiometry of an Indo-1–loaded mouse islet by confocal UV-laser microscopy. A confocal image of fluorescence-labeled insulin-containing cells in the same confocal plain (Ins.) was also shown. Outlines of insulin-negative cells and the islet were superimposed on the fourth pseudocolor image (15 min). The scale bar is 50 µm. *B:* A histogram of fluorescence intensity of the same islet after the immunocytochemical staining. Cells that had higher fluorescent intensity than 100 units (arrow) were regarded as insulin-positive. *C*: Time courses of $[Ca^{2+}]_c$ changes recorded every 10 s in the five cells. Positions of sampled cells are indicated in the fifth image in *A*. The arrows indicate the moments that the images depicted in *A* were recorded.

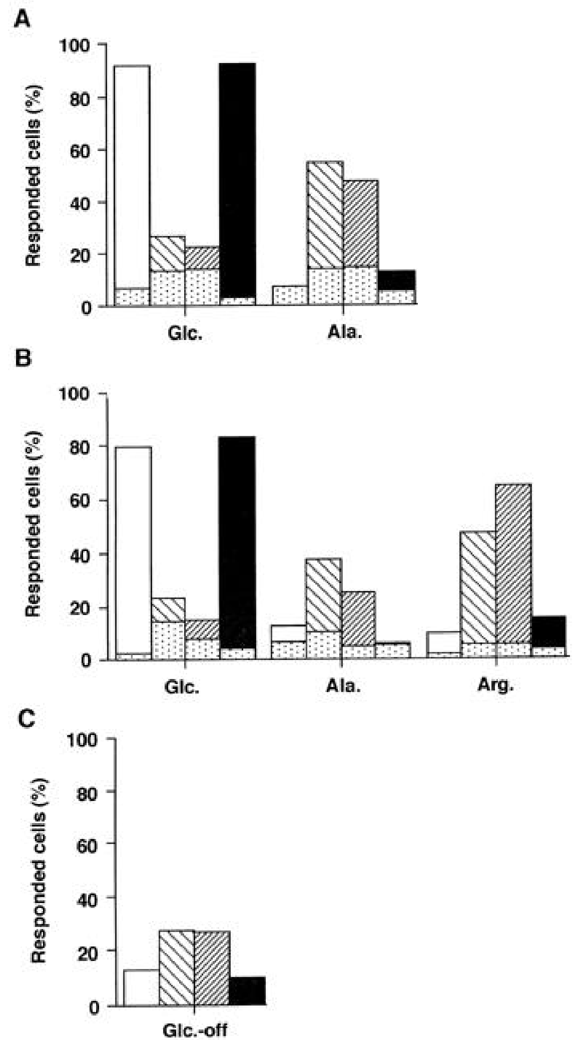


FIG. 2. Frequency of responsive cells to a change of glucose concentration or to the addition of amino acid in islet cells of the mouse. Cells whose $[Ca^{2+}]_c$ increased >50 nmol/l after treatments were judged as responsive cells. Frequency of cells with weak responses (amplitude of $[Ca^{2+}]_c$ rises <100 nmol/l) is expressed by the dotted columns in *A* and *B*. *A:* Results obtained from experiments at 34°C. *B:* Results obtained from experiments at 26°C. *C:* Frequency of cells that revealed glucose-off response at 34°C. □, insulin-positive cells; ▧, insulin-negative cells; ▨, glucagon-positive cells; ■, glucagon-negative cells.

recurrent rises in $[Ca^{2+}]_c$, which were inhibited after removal of glucose. The temporal sequences of $[Ca^{2+}]_c$ responses in individual glucagon-negative cells synchronized in the same islet during a continuous stimulation with 11 mmol/l glucose (cells c and d in Fig. 3 and Table 1). In total, 92.2% of glucagon-negative cells (248/269 cells) and 22.2% (12/54 cells) of α-cells were responsive to the increase in the glucose concentration (Fig. 2). A $[Ca^{2+}]_c$ increase was observed also in 13.1% of β-cells after glucose deprivation, but the rate of $[Ca^{2+}]_c$ rise was very slow, and the amplitude of the response was about a half of that observed in α-cells (Table 1).

**Amino acid–induced $[Ca^{2+}]_c$ response in β- and α-cells in intact islets.** When the islet was perifused with a solution containing 5 mmol/l alanine and 3 mmol/l glucose, β-cells did not respond to alanine (cells b and c in Fig. 1), but some insulin-negative cells (cells a and e) responded with $[Ca^{2+}]_c$ rises. The rate and amplitude of the $[Ca^{2+}]_c$ rises in insulin-negative cells were compatible to those in β-cells in response to the increase in glucose concentrations (Table 1). About one-half of insulin-negative cells (12/22 cells) and α-cells (10/21 cells) were responsive to the addition of 5 mmol/l alanine to the basal solution containing 3 mmol/l glucose in total (Fig. 2*A*). On the other hand, <7% of β-cells (6/87 cells) and 13.7% of glucagon-negative cells (22/161 cells) were responsive to alanine, and the $[Ca^{2+}]_c$ rise in β-cells was weaker than that

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TABLE 1
Comparison of rate and amplitude of $[Ca^{2+}]_c$ increase in β- and α-cells to various stimulations

| | β-Cells (*n*) | | α-Cells (*n*) | |
|---|---|---|---|---|
| | Rate (nmol · $l^{-1}$ · $min^{-1}$) | Amplitude of $[Ca^{2+}]_c$ increase (nmol · $l^{-1}$) | Rate (nmol · $l^{-1}$ · $min^{-1}$) | Amplitude of $[Ca^{2+}]_c$ increase (nmol · $l^{-1}$) |
| High glucose | 284.68 ± 17.45 | 449.22 ± 28.39 (31) | 87.77 ± 21.10 | 146.57 ± 32.76 (6) |
| Glucose deprivation | 30.54 ± 4.01 | 168.94 ± 23.66 (9) | 232.56 ± 72.10 | 334.06 ± 92.95 (5) |
| Alanine (3–5 mmol/l) | 51.20 ± 11.13 | 103.57 ± 20.61 (7) | 216.44 ± 50.32 | 230.39 ± 42.27 (5) |
| Arginine* (5–10 mmol/l) | 87.19 ± 16.95 | 126.43 ± 20.19 (7) | 250.04 ± 31.99 | 245.09 ± 18.93 (18) |

Data are means ± SE for the number of cells shown in parentheses. *Arginine results were obtained from experiments at 26°C.

in α-cells in response to alanine (Table 1). An insulin-negative cell (cell d in Fig. 1) responded to neither alanine nor glucose.

Sensitivity of β-cells to the increase in glucose concentration and that of α-cells to the addition of amino acids were little affected by lowering the temperature of the perifusion solution to 26°C (Fig. 2*B*). In the islet shown in Fig. 4, 24 of 32 glucagon-negative cells responded to the continuous stimulation with 15 mmol/l glucose by a sustained increase in $[Ca^{2+}]_c$, which decreased after the glucose concentration was restored to the basal level (3 mmol/l). Glucagon-negative cells showed little or no response to an addition of 10 mmol/l arginine to the basal solution containing 3 mmol/l glucose (cells b and e in Fig. 4). α-Cells in the islet did not respond to the solution containing 11 mmol/l glucose (Fig. 4*A*), but 12 of 16 α-cells responded to arginine with a rapid $[Ca^{2+}]_c$ rise (cells a, c, and d, for example). In total, 64.8% of α-cells (35/54 cells), 47.4% of insulin-negative cells (9/19 cells), 9.6% of β-cells (5/52 cells), and 14.8% of glucagon-negative cells (33/223 cells) were responsive to the addition of arginine (5–15 mmol/l) to the solution containing 3 mmol/l glucose at 26°C (Fig. 2*B*).

Stimulatory effects of amino acids on insulin secretion from the perifused pancreas are known to depend on glucose concentrations (26,27). When the glucose concentration in the perifusion solution was increased to 7 mmol/l, which is moderate stimulation for insulin secretion, arginine (7.5 mmol/l) increased $[Ca^{2+}]_c$ in most cells that responded to an increase in glucose (Fig. 5). Similar responses were observed when alanine (5 or 7.5 mmol/l) instead of arginine was added to the 7 mmol/l glucose solution.

Non–β-cells or α-cells often responded to an increase in glucose concentration with a $[Ca^{2+}]_c$ decrease when these cells exhibited a high $[Ca^{2+}]_c$. In total, only 8.9% of α-cells had such a high $[Ca^{2+}]_c$ in the basal solution containing 3 mmol/l glucose. $[Ca^{2+}]_c$ in those cells gradually decreased during perifusion with the solution containing high glucose (data not shown). It should be noted that the $[Ca^{2+}]_c$ of those cells consistently decreased in response to an increase in the glucose concentration when $[Ca^{2+}]_c$ was raised by glucose or amino acids. For example, $[Ca^{2+}]_c$ in one α-cell (cell c in the islet shown in Fig. 4) decreased during perifusion with the solution containing 15 mmol/l glucose and then remained stable at a lower level after the return to the original glucose concentration. In one insulin-negative cell (cell a in Fig. 1), $[Ca^{2+}]_c$ was stable at a high level after the stimulation by alanine (5 mmol/l) decreased in response to an increase in the glucose concentration. In total, ~85% of α-cells and ~70% of non–β-cells, whose $[Ca^{2+}]_c$ was stable at a high level at rest or after the stimulation with amino acids, responded to the increase in glucose concentration with a decrease in $[Ca^{2+}]_c$.

## DISCUSSION

In the present study, we for the first time demonstrated dynamic $[Ca^{2+}]_c$ changes in individual α- and β-cells in intact pancreatic islets by the combined use of Indo-1 ratio imaging and immunocytochemistry techniques with laser-scanning confocal microscopy. To date, there are several reports of $[Ca^{2+}]_c$ changes in β-cells in intact islets (13–16) as these cells consist of the majority in the islets; however, no report has been available concerning $[Ca^{2+}]_c$ changes in non–β-cells in the islet. This seems to be due to the difficulty in identifying individual cells in the islet with a conventional $Ca^{2+}$ imaging apparatus and because there are fewer α-cells and other non–β-cells than β-cells.

**The response to an increase in the glucose concentration.** The responses of pancreatic islet cells to an increase in concentrations of glucose were examined by recording Indo-1 ratio images and subsequent immunocytochemical staining with anti-insulin or anti-glucagon antibody after the recording. When experiments were performed at 34°C, >90% of β-cells and of glucagon-negative cells responded to an increase in the glucose concentration with oscillatory rises in $[Ca^{2+}]_c$. On the other hand, some α-cells and insulin-negative cells showed an increase in $[Ca^{2+}]_c$ in response to the increase in the glucose concentration. However, the response was relatively small in amplitude, slow in onset, and nonoscillatory (as seen in cell b in Fig. 1), and moreover, the $[Ca^{2+}]_c$ response was observed only in less than a quarter of such cells. These results indicate that the cells that responded to an increase in the glucose concentration with robust oscillatory $[Ca^{2+}]_c$ changes are β-cells. This is in agreement with several previous reports on $[Ca^{2+}]_c$ measurements in single β-cells (9–11), clusters of islet cells (12), or isolated islets (13–16). The $[Ca^{2+}]_c$ response persisted in ~80% of β-cells and glucagon-negative cells at lower temperature (26°C), but the oscillatory response observed at 34°C became a sustained increase on which small $[Ca^{2+}]_c$ fluctuations were often superimposed. These results are consistent with our previous results on the temperature-dependency of the glucose-induced $[Ca^{2+}]_c$ dynamics obtained from isolated pancreatic islets of rats (28). Although we have no firm explanation for the small $[Ca^{2+}]_c$ increase observed in some α-cells in response to glucose, it could be a result of some indirect action on α-cells induced by an increase in the glucose concentration. For example, the $[Ca^{2+}]_c$ rise in α-cells could be mediated through

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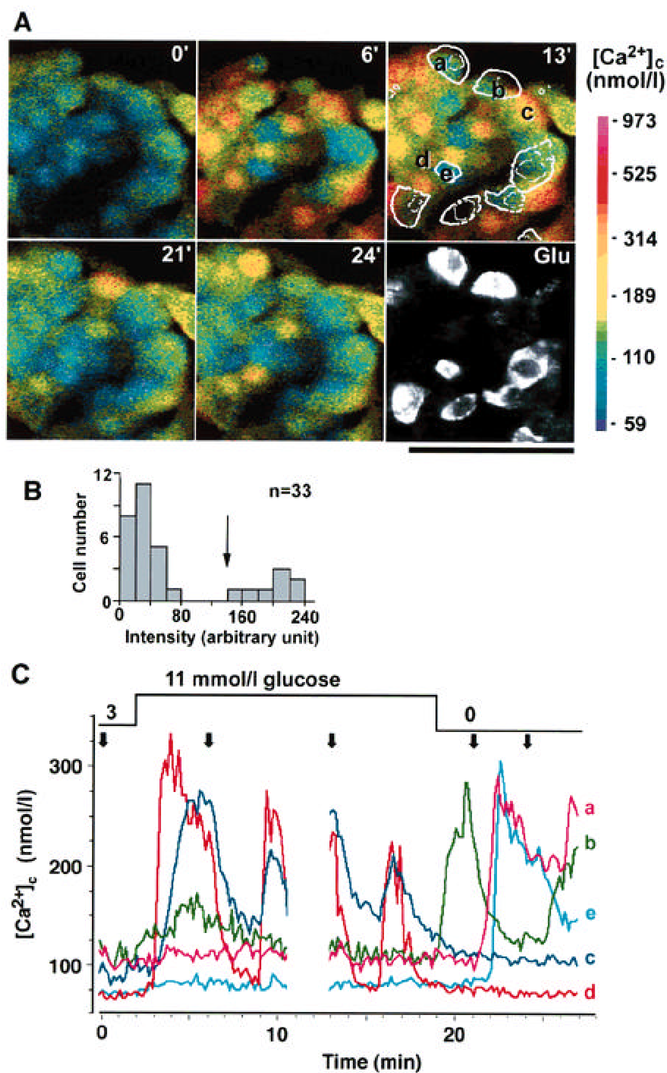


FIG. 3. $[Ca^{2+}]_c$ responses and glucagon immunofluorescence in an islet perifused with 11 mmol/l glucose followed by a glucose depletion at 34°C. *A:* Pseudocolor images of the spatial and temporal $[Ca^{2+}]_c$ changes and a confocal image of fluorescence-labeled glucagon containing cells in the same confocal plain (Glu). Outlines of glucagon-containing cells were superimposed on the third pseudocolor image (13 min). The scale bar is 50 µm. *B:* A histogram of fluorescence intensity of the same islet after the immunocytochemical staining. Cells that had a fluorescent intensity higher than 140 units (arrow) were regarded as glucagon-positive. *C:* Time courses of $[Ca^{2+}]_c$ changes recorded every 10 s in the five cells; positions of the sampled cells are indicated in third image in *A*. The arrows indicate the moments that the images depicted in *A* were recorded.

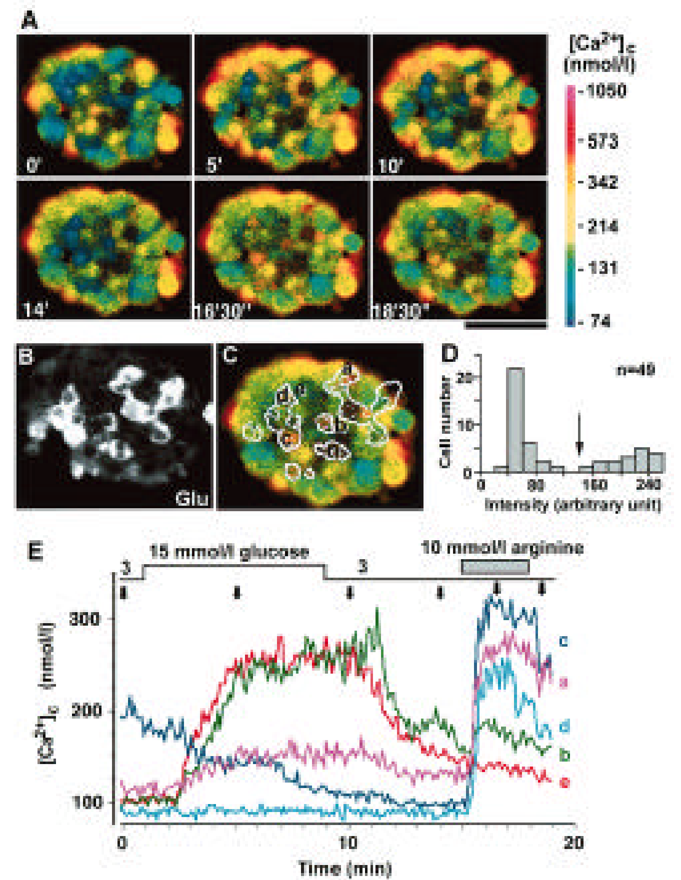


FIG. 4. $[Ca^{2+}]_c$ responses and glucagon immunofluorescence in an islet perifused with 15 mmol/l glucose followed by 10 mmol/l arginine at 26°C. The perifusion solution contained 3 mmol/l glucose throughout the experiment. *A*: Pseudocolor images showing the spatial and temporal $[Ca^{2+}]_c$ changes. The scale bar is 50 µm. *B*: A confocal image of fluorescence-labeled glucagon-containing cells in the same confocal plain of the islet (Glu). *C*: Outlines of glucagon-containing cells and the islet were superimposed on the pseudocolor image of the islet stimulated by arginine. Some of islet cells fell off or moved toward out-of-focus sites during the immunocytochemical procedure and therefore do not appear in the immunofluorescence image. *D*: A histogram of fluorescence intensity of the same islet after the immunocytochemical staining. Cells that had fluorescent intensity higher than 140 units (arrow) were regarded as glucagon-positive. *E*: Time courses of $[Ca^{2+}]_c$ changes recorded every 10 s in the five cells. Positions of sampled cells are indicated in *C*. The arrows indicate the moments that the images depicted in *A* were recorded.

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release of some intercellular signals that stimulate the neighboring α-cells in the islets, because the $[Ca^{2+}]_c$ rise in α-cell was synchronous to the initial rapid $[Ca^{2+}]_c$ rise observed in β-cells in the same islet (see, for example, Fig. 3*A* and *C*). The majority of α-cells and insulin-negative cells did not show a large increase in $[Ca^{2+}]_c$ in response to an increase in glucose concentration. Among such cells, a high percentage of cells with a high basal $[Ca^{2+}]_c$ responded to an increase in glucose concentration with a decrease in $[Ca^{2+}]_c$ (~85% of α-cells and 70% of insulin-negative cells possessing a high basal $[Ca^{2+}]_c$). Studies in isolated α-cells reported that $[Ca^{2+}]_c$ at a resting state or $[Ca^{2+}]_c$ that had been elevated by arginine (10 mmol/l) was lowered when the glucose concentration was increased from 0 to 20 mmol/l (17,18). Our results agree well with the effect of increasing the glucose concentration on elevated $[Ca^{2+}]_c$. However, in the present study, the basal $[Ca^{2+}]_c$ in α-cells did not respond to increasing glucose with a clear decrease. The difference between these and the present results could be due to the difference in the basal glucose concentration (we used 3 mmol/l glucose instead of 0 mmol/l) or to the difference in the preparations. In fact, some differences in hormone secretion from isolated cells and cells in islets have been reported (29,30). It is possible that under the basal condition, our preparation receives more inhibitory inputs than do isolated α-cells, considering that isolated α-cells are reported to exhibit $Ca^{2+}$ oscillations in 3 mmol/l glucose (19), whereas we did not observe such activity in α-cells

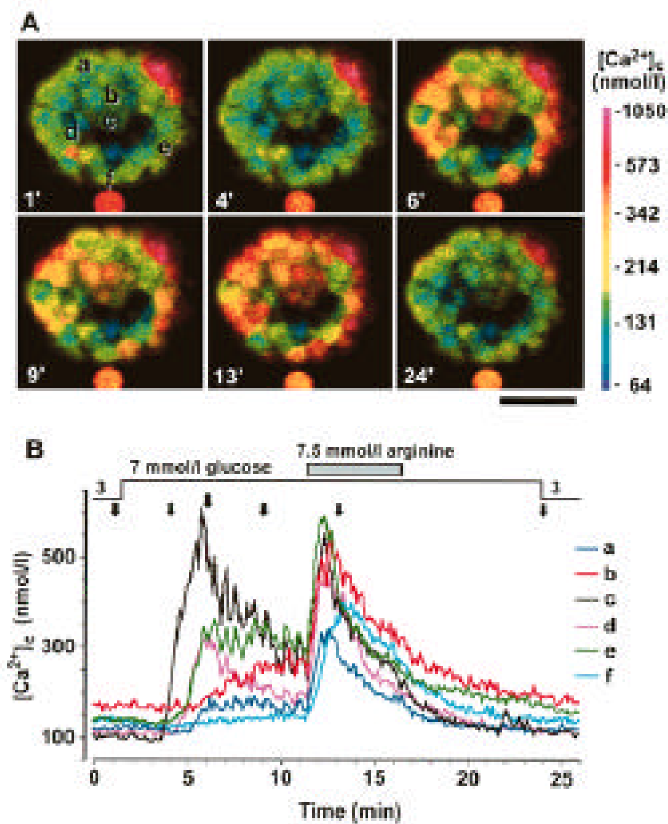


**FIG. 5.** $[Ca^{2+}]_c$ **responses in an islet perifused with 7 mmol/l glucose followed by addition of 7.5 mmol/l arginine at 26°C. The perifusion solution contained 3 mmol/l glucose throughout the experiment.** ***A*****: Pseudocolor images showing the spatial and temporal** $[Ca^{2+}]_c$ **changes in the confocal plain within the islet. The scale bar is 50 μm.** ***B:*** **Time courses of** $[Ca^{2+}]_c$ **changes recorded every 10 s in the six cells. Positions of sampled cells are indicated in the first image in *A*. The arrows indicate the moments that the images depicted in *A* were recorded.**

in 3 mmol/l glucose. In any event, the reduction in $[Ca^{2+}]_c$ observed in α-cells in response to the increase in the glucose concentration may account for, at least in part, the glucose-induced inhibition of glucagon secretion (26,27,31,32).

**Glucose-off response.** Although we did not observe oscillatory changes in $[Ca^{2+}]_c$ from glucagon-positive cells when islets were incubated in 3 mmol/l glucose (19), 27% of α-cells exhibited large and oscillatory increases in $[Ca^{2+}]_c$ when the glucose concentration was decreased from 11 to 0 mmol/l (glucose-off response). The $[Ca^{2+}]_c$ responses may account for the mechanism of glucagon secretion stimulated by decreasing glucose concentration, as the glucagon secretion is likewise dependent on an increase of intracellular $Ca^{2+}$ (34–36). Voltage-gated $Ca^{2+}$ channels may be involved in the responses, since electrophysiological analysis of $\alpha_2$-cells of guinea pig—selected by the autofluorescence cell sorting and morphological feature of cultured cells—revealed that these cells possess two types of $Ca^{2+}$ channels, L- and T-type $Ca^{2+}$ channels (37). The low percentage of large $[Ca^{2+}]_c$ responses to lowering glucose concentration in α-cells shows that glucose deprivation alone can only be a partial agonist for glucagon secretion. The increase in glucagon secretion from the perifused islets in response to the glucose-free solution was reported to be less than one-tenth of that in response to 20 mmol/l arginine in a glucose-free solution (32). The relatively weak response in glucagon secretion is consistent with observations in this study. On the other hand, glucagon secretion is also stimulated by adrenaline, and intracellular cAMP is thought to be the messenger for this stimulation (31,39,40). Further, it has been reported that an increase in cAMP concentration in α-cells releases $Ca^{2+}$ from the intracellular $Ca^{2+}$ stores and increases the sensitivity of glucagon release for $[Ca^{2+}]_c$ (36,39–41). Taken together, these results suggest that glucagon secretion of α-cells in vivo is regulated by factors that elevate cAMP, such as adrenaline, as well as by glucose concentration.

In 87% of insulin-positive cells, the $[Ca^{2+}]_c$ increased during stimulation with a high glucose concentration rapidly returned to the prestimulation level in a few minutes upon reduction in glucose concentration. In the remaining 13% of insulin-positive cells, however, $[Ca^{2+}]_c$ stayed at a high level or $[Ca^{2+}]_c$ oscillations lasted for several minutes, even after lowering glucose concentration. Because such $[Ca^{2+}]_c$ responses have not been observed in isolated β-cells, the residual $[Ca^{2+}]_c$ response could also be explained by some indirect action in islets that was triggered by lowering glucose concentration. The response could be correlated with the $[Ca^{2+}]_c$ rising response observed in α-cells when glucose concentration was suddenly lowered after a certain period of a high glucose concentration or after stimulation with amino acids.

**The response to amino acids.** When islets were perifused with a solution containing 3 mmol/l glucose and 5 mmol/l alanine at 34°C, 47.6% of α-cells showed $[Ca^{2+}]_c$ increase (Fig. 2). When the temperature was lowered to 26°C, the $[Ca^{2+}]_c$ increase in response to alanine was seen in only 25% of α-cells, and the $[Ca^{2+}]_c$ response to arginine (5–10 mmol/l) was 64.8%. The percentage of the cells that responded and the pattern of the $[Ca^{2+}]_c$ response are similar to those observed in α-cells in response to glucose deprivation (Table 1). It is reported that alanine or arginine stimulates glucagon release from perfused pancreas (26,27,29), isolated islets (29,32), or from isolated glucagon cells (39). Pipeleers et al. (39) also reported that arginine showed the strongest glucagon-releasing effects. Rises in $[Ca^{2+}]_c$ in response to arginine have been reported in single α-cells of guinea pigs (17) and rats (18). Such close similarity between the $[Ca^{2+}]_c$ response observed in α-cells and glucagon release suggests that the $[Ca^{2+}]_c$ response accounts for amino-acid–induced glucagon release from α-cells.

Alanine and arginine also stimulate insulin release from the β-cells (26,29,30,42–44) through the depolarization of the cellular membrane (45) and were reported to increase $[Ca^{2+}]_c$ in isolated β-cells (46) or in whole islets (47). However, the stimulatory effect of these amino acids on the β-cells depends on the glucose concentrations of extracellular space. Insulin secretion and membrane depolarization induced by amino acids were abolished or diminished in a low-glucose solution or in a glucose-free solution (26,30,31,45). Thus, the addition of amino acids to the solution containing 3 mmol/l glucose partially stimulated the $[Ca^{2+}]_c$ increase in the β-cells in the intact islets. Indeed, arginine (7.5 mmol/l) increased $[Ca^{2+}]_c$ in most islet cells when it was added to the solution containing 7 mmol/l glucose (Fig. 5), and this is comparable to $[Ca^{2+}]_c$ responses in the intact islets stimulated by the solution containing 11 mmol/l glucose and amino acids (48,49).

In conclusion, the present study provided a technique to investigate $[Ca^{2+}]_c$ dynamics in individual cells within intact islets and clarified $[Ca^{2+}]_c$ responses in α- and β-cells in whole

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islets in response to glucose or amino acids. β-Cells in the islet showed large and repetitive increases in $[Ca^{2+}]_c$ in response to increases in the glucose concentration and little or no response to amino acids in 3 mmol/l glucose solution. On the contrary, α-cells in the islet responded to both glucose deprivation and the addition of amino acids to 3 mmol/l glucose solution with an increase in $[Ca^{2+}]_c$.

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