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Measurement of Glucose Uptake and Intracellular Calcium Concentration in Single, Living Pancreatic β-Cells*

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There has been no method previously to measure both glucose transport and its effect on the various intracellular functions in single, living mammalian cells. A fluorescent derivative of D-glucose, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG), that we have developed has made such measurements possible. COS-1 cells that overexpress the human glucose transporter GLUT2 show significantly greater 2-NBDG uptake than mock transfected cells. Using GLUT2-abundant mouse insulin-secreting clonal MIN6 cells, we found that 2-NBDG was incorporated into the cells in a time- and concentration-dependent manner. The 2-NBDG uptake was inhibited by high concentrations of D-glucose in a dose-dependent manner and also was almost completely inhibited by 10 μM cytochalasin B. We then measured both glucose uptake and the intracellular calcium concentration ($[Ca^{2+}]_i$) in single, living pancreatic islet cells. 2-NBDG and fura-2 were used as the tracer of glucose and indicator of intracellular calcium, respectively. All of the cells that showed an increase in $[Ca^{2+}]_i$ in response to a high concentration of glucose (16.8 mM) rapidly incorporated significant 2-NBDG. Immunocytochemical examination confirmed these cells to be insulin-positive β-cells. All of the cells that showed no significant, rapid 2-NBDG uptake lacked such glucose responsiveness of $[Ca^{2+}]_i$, indicating that these cells were non-β-cells such as glucagon-positive α-cells. These results show the uptake of glucose causing a concomitant increase of $[Ca^{2+}]_i$ in β-cells. Because 2-NBDG is incorporated into mammalian cells through glucose transporters, it should be useful for the measurement of glucose uptake together with concomitant intracellular activities in many types of single, living mammalian cells.

Glucose transport activity in mammalian cells has been monitored by radiolabeled tracers such as [^{14}C] 2-deoxy-D-glucose (1), [^{18}F] fluoro-2-deoxy-D-glucose (2), and [^{14}C] or [^{3}H]3-*O*-methyl-D-glucose (3, 4). Although these methods are quite effective in glucose utilization studies (5–7), they cannot measure glucose uptake in single, living cells.

We have recently developed a fluorescent D-glucose derivative, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG)[1] (8), that allows a more sensitive measurement of glucose uptake in real time in single, living cells. Another advantage of measuring glucose uptake by the 2-NBDG molecule is that it allows concomitant measurement of other cellular activities, such as the intracellular calcium concentration ($[Ca^{2+}]_i$), pH, or membrane potential by different methods. However, it has not been established that 2-NBDG is incorporated into mammalian cells, in which glucose is transported by facilitated diffusion through a family of glucose transporters GLUTs (9).

Among the several GLUTs identified to date, GLUT2 has high K_m for glucose and is abundant in cells that sense glucose, such as pancreatic β-cells (9). It is thought that when the blood glucose level is elevated, glucose uptake into the pancreatic β-cells through GLUT2 is increased and that this incorporated glucose is then metabolized within the cell. Among the various metabolic products, ATP is an essential molecule for insulin secretion (10); an increase in the intracellular ATP concentration in the β-cell closes the ATP-sensitive K^+ channel, leading to plasma membrane depolarization and influx of Ca^{2+} through the voltage-gated calcium channels. The subsequent rise in $[Ca^{2+}]_i$ triggers insulin secretion. However, the correlation between glucose uptake and changes in $[Ca^{2+}]_i$ in response to glucose stimulation has not been shown directly in single insulin-secreting β-cells.

To determine whether living, mammalian cells incorporate 2-NBDG through GLUT, we examined 2-NBDG uptake activity of cells in which GLUT2 is overexpressed or abundant, using fluorescence microscopy. We monitored glucose uptake activity and the $[Ca^{2+}]_i$ level in single, living pancreatic islet cells using 2-NBDG as a tracer of glucose and fura-2 as an intracellular calcium indicator. Our results suggest that 2-NBDG should be useful in analysis of the mechanisms underlying glucose uptake and concomitant cellular functions in mammalian cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Culture and transfection of COS-1 cells was carried out as described previously (11). Briefly, COS-1 cells were plated on 35-mm culture dishes at a density of 2×10^5 cells/dish 24 h prior to transfection and cultured in Dulbecco's modified Eagle's medium (450 mg/dl glucose) supplemented with 10% fetal calf serum. Two micrograms of human GLUT2 expression vector (pCMVGLUT2) was transfected into COS-1 cells with LipofectAMINE and Opti-MEM I (Life Technologies, Inc.), according to the manufacturer's instructions. As control, COS-1 cells transfected with vector (pCMV) alone were used. MIN6 cells were cultured in the same medium as COS-1 cells.

Preparation of Rat Islet Cells—Islets of Langerhans were isolated from 8–12-week-old Harlan Sprague-Dawley rats by collagenase diges-

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[1] The abbreviations used are: 2-NBDG, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose; KRB, Krebs Ringer bicarbonate buffer; DNP, 2,4-dinitrophenol.

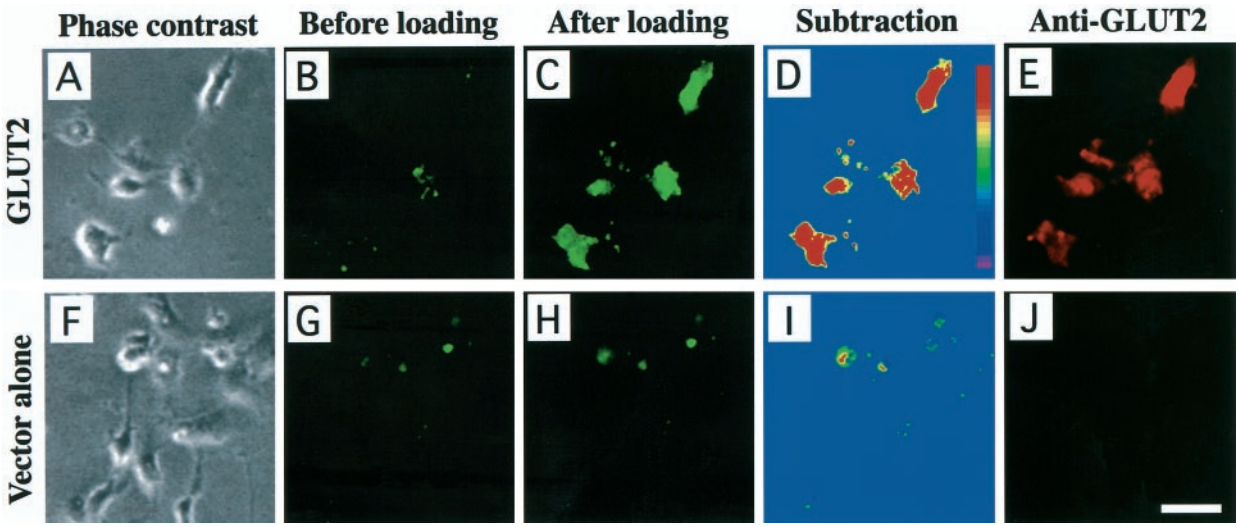

FIG. 1. **Comparison of 2-NBDG uptake of GLUT2-overexpressing COS-1 cells and mock transfected COS-1 cells.** Population of COS-1 cells transfected with GLUT2 expression vector (*A–E*) (5 cells) or vector alone (*F–J*) (10 cells). *A* and *F*, phase contrast images of cells. *B* and *G*, fluorescent images measured at 540-nm wavelength before loading 2-NBDG. *C* and *H*, fluorescent images measured at 540 nm 5 min after loading 200 μM 2-NBDG. *D* and *I*, net increase in the 2-NBDG fluoresence in cells shown as pseudocolor subtraction images. Fluorescence intensity before application of 2-NBDG was subtracted from that after its application. The *bottom* of the *color scale bar* indicates low fluorescence intensity and the top high intensity. *E* and *J*, immunocytochemistry of GLUT2. Cells were incubated with GLUT2 primary antibody and rhodamine-conjugated secondary antibody. In *B*, *C*, *G*, and *H*, cells were superfused continuously with KRB containing 5.6 mM glucose at 25 °C. *Scale bar* is 50 μm.

tion as described previously (12). Isolated islets were dissociated into single cells by incubation in Ca^{2+}-free Krebs Ringer bicarbonate buffer (KRB) containing 1 mM EGTA but no added Ca^{2+}. KRB was composed of 129 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.0 mM $CaCl_2$, 5.0 mM $NaHCO_3$, and 10 mM HEPES, pH 7.4, supplemented with 0.1% bovine serum albumin. After centrifugation, the single cells were resuspended in Eagle's minimum essential medium supplemented with 10% fetal bovine serum and 60 μg/ml kanamycin, plated on coverslips, and cultured up to 3 days at 37 °C in a humidified atmosphere containing 5% CO_2.

Measurement of 2-NBDG Uptake—The cells were mounted in a chamber and placed on the stage of an inverted microscope and superfused with KRB containing various concentrations of 2-NBDG at a flow rate of 0.3 ml/min (12). Delivery and removal of superfusate was by peristaltic or vacuum pump. Fluorescence of 2-NBDG was collected by a silicon intensified target camera at 520–560-nm wavelength (excitation wavelength 465–495 nm). Images of 15–100 cells/each preparation were digitized in 8 bit, and integrated 16 or 64 times at video rate by an Argus-50 system (Hamamatsu Photonics, Hamamatsu, Japan). After background subtraction, fluorescence intensity was calculated as the difference in the average fluorescence of cells before and after application of 2-NBDG. Adequate neutral density filters were selected so that 2-NBDG fluorescence intensity was in the linear range of fluorescence *versus* concentration of 2-NBDG. In time and temperature dependence experiments, MIN6 cells (13) were superfused in D-glucose-free KRB for 15 min, and then the superfusate was changed to D-glucose-free KRB containing 600 μM 2-NBDG. After superfusion for the periods indicated at 25 or 37 °C, the cells were washed for 5 min, and the fluorescence images were collected. Concentration dependence experiments were carried out under the same condition except that the cells were superfused for 1 min at 37 °C in D-glucose-free KRB containing the concentrations of 2-NBDG indicated. Similarly, the effect of D-glucose on 2-NBDG uptake was estimated by incubating the cells for 1 min at 37 °C in KRB containing 600 μM 2-NBDG in the presence of the concentrations of D-glucose indicated. The effects of cytochalasin B and phloretin on 2-NBDG uptake were examined by superfusing the cells for 15 s with 200 μM 2-NBDG in the presence of 5.6 mM D-glucose at 25 °C; the experiments on overexpression of GLUTs in COS-1 cells were also performed under the same condition.

Measurement of $[Ca^{2+}]_i$ and 2-NBDG Uptake—The islet cells were incubated with 1 μM fura-2/acetoxymethylester (14) for 30 min at 37 °C in KRB containing 2.8 mM glucose. The cells were then mounted in the chamber and superfused with KRB containing a basal (2.8 mM) or elevated (16.8 mM) concentration of glucose at 37 °C as described above. Fura-2 fluorescence was detected by a silicon intensified target camera every 5 or 10 s at 500–520-nm wavelength following excitation at 340-nm (F340) and 380-nm (F380) wavelengths, and the ratio image (F340/F380) was calculated by an Argus-50. After measurement of $[Ca^{2+}]_i$, 2-NBDG uptake into the same cells was estimated by superfusion at 37 °C for 1 min in KRB containing 200 μM 2-NBDG and 2.8 mM D-glucose. In some experiments, the glucose-induced $[Ca^{2+}]_i$ response and 2-NBDG uptake were measured in the presence of 50 μM of 2,4-dinitrophenol (DNP). Because DNP has an effect of elevating basal $[Ca^{2+}]_i$ (15), high glucose was applied when this elevation was stabilized.

Immunocytochemistry—After measuring the fluorescence of 2-NBDG and $[Ca^{2+}]_i$, COS-1 cells or islet cells were fixed in 0.1 M sodium phosphate buffer containing 4% paraformaldehyde and pretreated with 1% bovine serum albumin before incubation with anti-GLUT2, anti-insulin, or anti-glucagon antibody. For staining of GLUT2, the cells were incubated with rabbit anti-mouse GLUT2 antibody (1: 400) (Chemicon International Inc., Temecula, CA) for 1 h at room temperature, followed by incubation with rhodamine-conjugated goat anti-rabbit IgG (1:500) (Cappel, West Chester, PA) for 20 min at room temperature. For staining β-cells, the cells were incubated with guinea pig anti-swine insulin antibody (1:100) (Dako Corp., Carpinteria, CA) at 4 °C overnight, followed by incubation with rhodamine-conjugated goat anti-guinea pig IgG (1:500) (Chemicon International Inc., Temecula, CA) for 1 h at room temperature. To stain α-cells, the cells were incubated with rabbit anti-porcine glucagon antibody (prediluted, Dako Corp., Carpinteria, CA) at 4 °C overnight, followed by incubation with rhodamine-conjugated goat anti-rabbit IgG (1:100) (Cappel, West Chester, PA) at room temperature for 1 h. Rhodamine fluorescence was examined by a silicon intensified target camera with a 590-nm longpass filter (excitation wavelength 510–560 nm).

RESULTS

2-NBDG Uptake through Mammalian Glucose Transporters—To determine whether 2-NBDG is transported through mammalian glucose transporters, we measured the uptake of 2-NBDG into cells overexpressing human GLUT2. The mammalian expression vector carrying GLUT2 cDNA (pCMV-GLUT2) or vector alone (pCMV) was transfected into COS-1 cells, and 48 h later the cells were loaded for 15 s with the medium containing 200 μM 2-NBDG, followed by washout. 5 min later, the fluorescence intensity of the cells was measured at 540-nm wavelength by fluorescence microscopy. COS-1 cells often showed granular autofluorescence in the cytosol before incubation in the presence of 2-NBDG (Fig. 1, *B* and *G*). After application of 2-NBDG, the fluorescence intensity of COS-1 cells transfected with GLUT2 expression vector was remarkably increased, and the cells can be more clearly discerned

(Fig. 1, *A–C*). The net increase in fluorescence intensity of the cells is shown in Fig. 1*D* as a subtraction image; the fluorescence intensity before application of 2-NBDG was subtracted from that after its application. On the contrary, COS-1 cells transfected with the vector alone showed no remarkable difference in fluorescence before or after application of 2-NBDG (Fig. 1, *F–I*). These results were confirmed in eight separate experiments.

To confirm that the COS-1 cells showing strong 2-NBDG fluorescence actually expressed abundant GLUT2, the cells were immunocytochemically stained with anti-GLUT2 antibody after measurement of 2-NBDG uptake. As shown in Fig. 1*E*, the COS-1 cells that emitted 2-NBDG fluorescence stained with anti-GLUT2 antibody, but the control cells did not (Fig. 1*J*). Uptake of 2-NBDG into COS-1 cells transfected with GLUT1 or GLUT3 also was increased compared with those of mock transfected COS-1 cells (data not shown).

To confirm that 2-NBDG is transported thorough glucose transporters, the time course and concentration dependence of 2-NBDG uptake were measured in MIN6 cells, mouse insulin-secreting clonal β-cells. MIN6 cells are known to exhibit glucose-inducible insulin secretion comparable with normal mouse islet cells and also to express GLUT2 at a high level but GLUT1 at a barely detectable level, as do mouse pancreatic β-cells (13). The MIN6 cells were superfused with 600 μM 2-NBDG in the absence of glucose for 15 s to 20 min at 37 °C followed by washing for 5 min, and the uptake of 2-NBDG was evaluated by fluorometry. As shown in Fig. 2*A*, the time course was almost linear up to 2 min, and 2-NBDG fluorescence intensity at 2 min approached 43% (mean of two independent experiments) of that at 20 min at 37 °C. The initial velocity of 2-NBDG uptake into the MIN6 cells, therefore, was determined by the difference in the fluorescence intensity before and after 1-min superfusion with 2-NBDG at 37 °C in the absence of glucose. It increased in a concentration-dependent manner (Fig. 2*B*), and Eadie-Hofstee transformation of these data resulted in a nonlinear curve with two kinetic components, apparent K_m values of 13.3 and 1.6 mM (Fig. 2*C*). 2-NBDG uptake was temperature-sensitive, and the initial velocity of the 2-NBDG uptake at 25 °C was approximately half that at 37 °C (Fig. 2*A*).

To further confirm that 2-NBDG is incorporated thorough glucose transporters, the effects of D-glucose and cytochalasin B, an antagonist of glucose transporters (4, 16, 17), on the uptake of 2-NBDG into MIN6 cells were examined. The cells were incubated with 600 μM 2-NBDG for 1 min in the absence or presence of various concentrations of D-glucose. 2-NBDG uptake was inhibited by D-glucose in a dose-dependent manner; it was inhibited by 37.9 ± 10.1, 52.5 ± 6.3, and 70.1 ± 7.8% (mean ± S.E. of three independent experiments) in the presence of 5.6, 11.2, and 22.4 mM D-glucose, respectively. Cytochalasin B was added to the medium 5 min prior to superfusion of the cells in the medium containing 2-NBDG. As seen in Fig. 3, the fluorescence intensity of the MIN6 cells that was increased after incubation with 200 μM 2-NBDG was almost completely inhibited in the presence of 10 μM cytochalasin B. These results were confirmed in six separate experiments. The increase in fluorescence intensity also was inhibited by 100 μM phloretin (data not shown). These results demonstrate that 2-NBDG is transported into cells through mammalian glucose transporters.

Measurement of 2-NBDG Uptake and $[Ca^{2+}]_i$—Glucose uptake activity and the glucose responsiveness of single pancreatic islet cells were measured. 2-NBDG was used as a tracer of glucose uptake, and changes in the $[Ca^{2+}]_i$ level in response to high glucose stimulation were monitored using fura-2 as an indicator.

A representative experiment is shown in Fig. 4. Seven of the

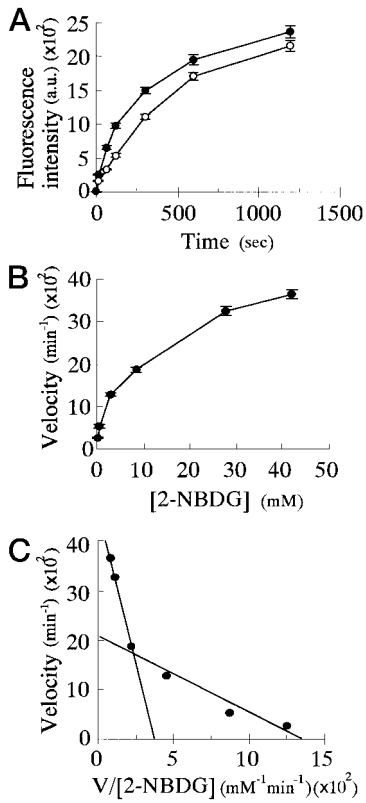

FIG. 2. **Kinetic analyses of 2-NBDG uptake into MIN6 cells.** *A*, time course of 600 μM of 2-NBDG uptake at 37 °C (*filled circles*) and 25 °C (*open circles*). Cells were superfused continuously with KRB. Fluorescence intensities were expressed as arbitrary units (*a.u.*) determined by fluorometry as described under "Experimental Procedures." Data are the means ± S.E. of 33–50 cells from an experiment representative of at least two independent experiments. *B*, concentration dependence of initial velocity of 2-NBDG uptake. Initial velocity of the uptake was estimated to be the difference in the fluorescence intensity before and 1 min after application of 2-NBDG at 37 °C. Data are the means ± S.E. of 27–42 cells from an experiment representative of two independent experiments. *C*, Eadie-Hofstee plot of 2-NBDG uptake activity. Best fitted lines were drawn by linear regression analysis of the data shown in *B*.

nine islet cells showed marked increases in $[Ca^{2+}]_i$ when the extracellular glucose concentration was transiently elevated from 2.8 to 16.8 mM (Fig. 4, *A* and *B*, cells 1–7). The $[Ca^{2+}]_i$ of these seven cells returned to basal level upon reversal of the glucose concentration to 2.8 mM (Fig. 4*B*). We then examined uptake of 2-NBDG into the same cells. The seven glucose-responsive cells emitted strong 2-NBDG fluorescence when 200 μM 2-NBDG was applied (Fig. 4, *A–E*, cells 1–7). On the contrary, the two other islet cells that showed no increase in $[Ca^{2+}]_i$ in response to a high concentration of glucose emitted no significant 2-NBDG fluorescence (Fig. 4, *A–E*, cells 8 and 9). Morphologically, the cells that showed no glucose responsiveness or 2-NBDG uptake appeared somewhat smaller than the glucose-responsive cells, suggesting that the glucose-responsive cells are β-cells and the unresponsive cells are non-β-cells (18). The same cells were then characterized immunocytochemically after measurement of $[Ca^{2+}]_i$ and 2-NBDG uptake. As shown in Fig. 4*F*, the seven glucose-responsive islet cells stained with anti-insulin antibody (cells 1–7), but the other two cells did not (cells 8, 9), further suggesting that only the cells showing both glucose responsiveness and 2-NBDG uptake are β-cells. We confirmed that all of the 93 cells that exhibited significant rapid 2-NBDG uptake were β-cells in 12 separate experiments. Most of these cells (82 cells) exhibited an increase

in $[Ca^{2+}]_i$ in response to glucose, but 11 of them did not, although they did respond to the sulfonylurea tolbutamide (0.5 mM) (data not shown).

To examine the effect of glucose metabolism on the glucose-induced $[Ca^{2+}]_i$ increase in β-cells, DNP, an uncoupler of mitochondrial oxidative phosphorylation (15, 19), was used. A representative experiment is shown in Fig. 5. The increase in $[Ca^{2+}]_i$ in response to 16.8 mM glucose was strongly inhibited in the presence of 50 μM DNP, but the responsiveness was restored after washout of DNP. The cells also responded to 0.5 mM tolbutamide, and the same cells emitted strong 2-NBDG fluorescence when 200 μM 2-NBDG was applied. These results were found in all glucose-responsive cells examined (34 cells) in five separate experiments. We also examined the effect of DNP on 2-NBDG uptake. 2-NBDG (200 μM) was applied to β-cells in the presence of 50 μM DNP, but 2-NBDG uptake into the β-cells was unaffected (five separate experiments; data not shown).

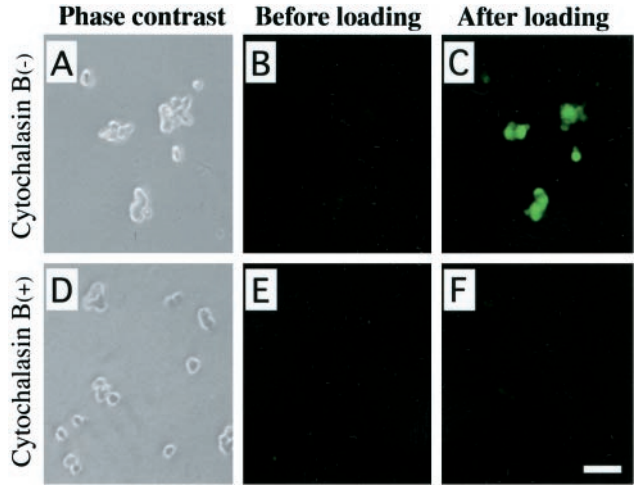

FIG. 3. **Effect of cytochalasin B on 2-NBDG uptake into MIN6 cells.** Clusters of MIN6 cells incubated in the absence (*A–C*) or presence (*D–F*) of 10 μM cytochalasin B are shown. *A* and *D*, phase contrast images of cells. *B* and *E*, fluorescent images measured at 540-nm wavelength before loading 2-NBDG. *C* and *F*, fluorescent images at 540 nm 5 min after loading 200 μM 2-NBDG without (*C*) or with (*F*) 10 μM cytochalasin B. In *B*, *C*, *E*, and *F*, cells were superfused continuously with KRB containing 5.6 mM glucose at 25 °C. *Scale bar* is 50 μm.

To ascertain the inability of non-β-cells to incorporate 2-NBDG, an experiment with glucose and tolbutamide was performed, as shown in Fig. 6. Three of the four islet cells showed a $[Ca^{2+}]_i$ increase in response to both 16.8 mM glucose and 0.5 mM tolbutamide and incorporated significant 2-NBDG (Fig. 6, *A–E*, cells 1–3). The one islet cell that exhibited no $[Ca^{2+}]_i$ increase in response to either glucose or tolbutamide also did not show significant 2-NBDG uptake and was stained with anti-glucagon antibody (Fig. 6, *A–F*, cell 4). We performed eight separate experiments, and nine of the thirteen cells that exhibited no glucose- or tolbutamide-induced $[Ca^{2+}]_i$ increase also incorporated no significant 2-NBDG rapidly and were stained with anti-glucagon antibody. The other four cells were not stained, indicating that they are non-β-, non-α-cells such as somatostatin-secreting δ-cells or pancreatic polypeptide-secreting PP cells. In contrast, no glucose- or tolbutamide-responsive cells were stained with anti-glucagon antibody (43 cells).

To ascertain that the non-β-cells incorporate 2-NBDG after longer incubation, 2-NBDG uptake into islet cells was estimated after 10-min superfusion with 200 μM 2-NBDG. In a representative experiment, all 95 of the islet cells eventually showed 2-NBDG uptake, although there was extremely weak flouresence intensity in a small population of the cells (18 cells). We determined that 10 of the 18 cells were α-cells by staining with anti-glucagon antibody, and the average fluorescence intensity of the α-cells (379.1 ± 37.4; mean ± S.E.) was about one-tenth that of β-cells measured under the same condition (3000–6000). These results were confirmed in three independent experiments.

DISCUSSION

2-NBDG is a fluorescent derivative of D-glucose that has been modified with a 2-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino group at the C-2 position. We have shown in previous studies that 2-NBDG is taken into the cytoplasm of both *Escherichia coli* cells and yeast *Candida albicans* cells and that it is

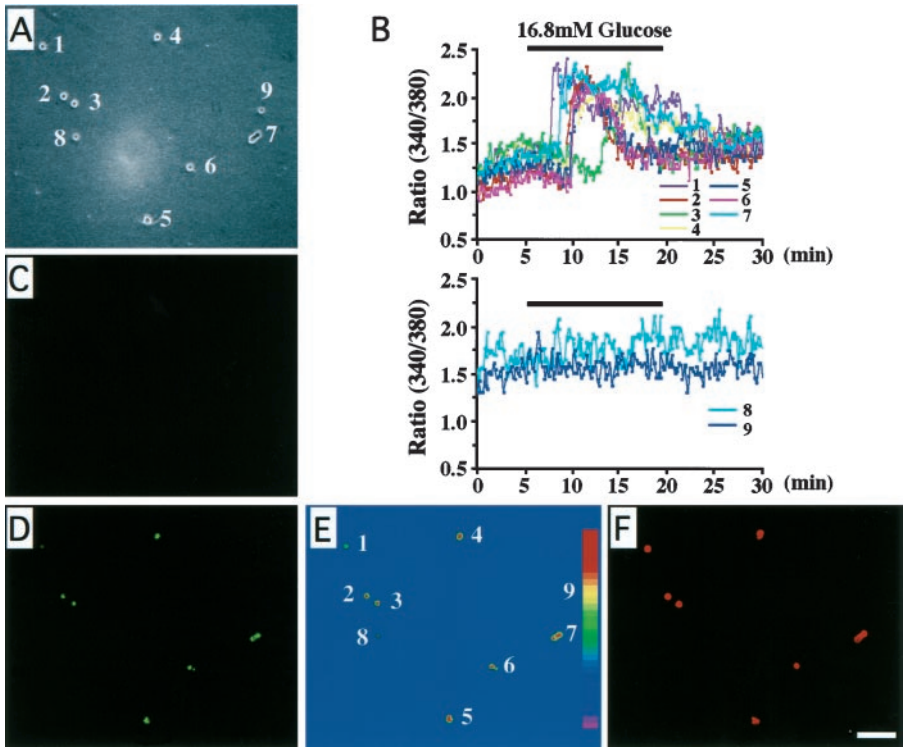

FIG. 4. **Measurement of 2-NBDG uptake and $[Ca^{2+}]_i$ in pancreatic islet cells.** Population of rat pancreatic islet cells (9 cells) was imaged. *A*, a phase contrast image. *B*, effects of high glucose (16.8 mM) on $[Ca^{2+}]_i$ in the islet cells. $[Ca^{2+}]_i$ responses are expressed as the change in fura-2 fluorescence ratio (340/380 nm). *Horizontal bars* indicate the periods of high glucose applications. *C*, a fluorescent image measured at 540-nm wavelength before loading 2-NBDG. *D*, a fluorescent image at 540 nm after loading 200 μM 2-NBDG for 1 min. *E*, net increase in 2-NBDG uptake into islet cells shown as a pseudocolor subtraction image. The *bottom* of the *color scale bar* indicates low fluorescence intensity and the top high intensity. *F*, immunocytochemistry of insulin. Cells were incubated with insulin primary antibody and rhodamine-conjugated secondary antibody. In *B–D*, cells were superfused continuously with KRB containing 2.8 mM glucose at 37 °C; the KRB was supplemented as indicated. *Numbers* identify the cells in *A–F*. *Scale bar* is 100 μm.

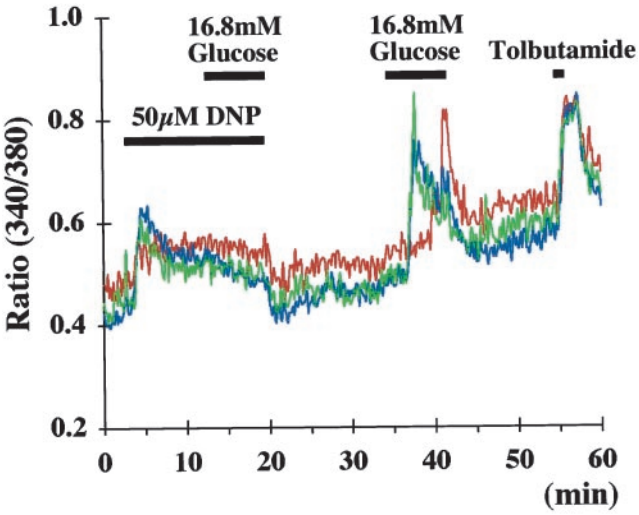

FIG. 5. **Effect of DNP on glucose-induced $[Ca^{2+}]_i$ change in islet cells.** Effects of high glucose (16.8 mM) and 0.5 mM tolbutamide on $[Ca^{2+}]_i$ in three islet cells in the absence or presence of 50 μM DNP. $[Ca^{2+}]_i$ responses are expressed as the change in fura-2 fluorescence ratio (340/380 nm). Cells were superfused continuously with KRB containing 2.8 mM glucose at 37 °C. *Horizontal bars* indicate the periods of DNP, high glucose, and tolbutamide applications.

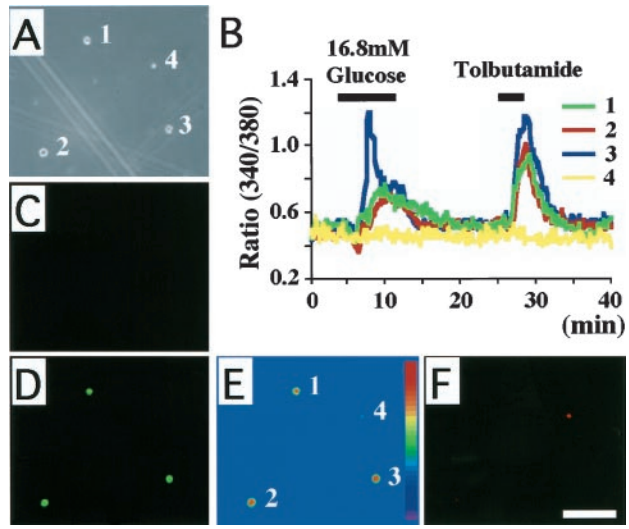

FIG. 6. **Characterization of glucose-unresponsive islet cells.** A population of rat pancreatic islet cells (4 cells) was imaged. *A*, a phase contrast image of the islet cells. *B*, effects of 16.8 mM glucose and 0.5 mM tolbutamide on $[Ca^{2+}]_i$ in the cells. $[Ca^{2+}]_i$ responses are expressed as the change in fura-2 fluorescence ratio (340/380 nm). *Horizontal bars* indicate the periods of high glucose and tolbutamide applications. *C*, a fluorescent image measured at 540-nm wavelength before loading 2-NBDG. *D*, a fluorescent image at 540 nm after loading 200 μM 2-NBDG for 1 min. *E*, net increase in 2-NBDG uptake into the cells shown as a pseudocolor subtraction image. The *bottom* of the *color scale bar* indicates low fluorescence intensity and the top high intensity. *F*, immunocytochemistry of glucagon. Only the glucose-unresponsive cell (cell4) was stained. Cells were incubated with glucagon primary antibody and rhodamine-conjugated secondary antibody. In *B–D*, cells were superfused continuously with KRB containing 2.8 mM glucose at 37 °C, and the KRB was supplemented as indicated. *Numbers* identify the cells in *A–F*. *Scale bar* is 100 μm.

useful for assaying viability of cells (8, 20). Uptake of 2-NBDG into these cells is inhibited by D-glucose but not by L-glucose, suggesting that it is transported into the cells through the glucose transporter system (8, 20), but this has not been determined in mammalian cells. In the present study, we have demonstrated that 2-NBDG is incorporated into mammalian cells through GLUTs. 2-NBDG was incorporated into the mouse insulin-secreting clonal MIN6 cells in a time- and concentration-dependent manner, and kinetic analysis revealed two K_m components of 13.3 and 1.6 mM. These values are similar to those for D-glucose and the nonmetabolizable glucose analogue, 3-*O*-methyl-D-glucose (17), found in pancreatic islets and cultured pancreatic β-cells, in which GLUT1 is expressed at low levels, whereas GLUT2 is much more abundant (21, 22). Because MIN6 cells also express GLUT1 at a very low level (13), these data suggest that the high K_m and low K_m correspond to the affinities of 2-NBDG for GLUT2 and GLUT1, respectively. Ishihara *et al.* (23) have reported that the uptake of 3-*O*-methyl-D-glucose is rapid and that equilibration is 80% complete in 1 min in MIN6 cells. This is comparable with the time course of 2-NBDG uptake into MIN6 cells reported in this study, although the 2-NBDG uptake is somewhat slower.

A fluorescent derivative of D-glucose, 6-NBDG, which is modified at the C-6 position, has been developed by Speizer *et al.* (24). They found 6-NBDG to be incorporated into human erythrocytes but gradually to come out of the cell without any modification, probably because it does not enter the glycolytic pathway, glucose metabolism beginning with phosphorylation at the 6-hydroxyl group of D-glucose. In contrast, 2-NBDG is metabolized to a phosphorylated fluorescent derivative at the C-6 position, *i.e.* 2-NBDG 6-phosphate, after incorporation, and then decomposes to a nonfluorescent derivative in *E. coli* cells (8, 25). Thus, the level of fluorescence intensity of 2-NBDG may be equilibrium of generation and decomposition of the fluorescent derivative. 2-NBDG fluorescence was not noticeably reduced up to 30 min after loading in this study (data not shown).

We then used 2-NBDG to evaluate glucose uptake activity together with changes in $[Ca^{2+}]_i$ in response to glucose stimulation in single, living pancreatic islet cells. It is thought that although an increase in glucose metabolism is essential for insulin secretion, the capacity for glucose uptake is very high, so transport is not rate-limiting. Gorus *et al.* (26) have separated β-cells from non-β-cells by fluorescence-activated cell sorting and shown that [^{14}C] D-glucose or 3-*O*-methyl-D-glucose is rapidly equilibrated within 2 min across the plasma membrane in β-cells at both low (600 μM) and high (20 mM) glucose concentrations. In contrast, they showed that intracellular [^{14}C] D-glucose or 3-*O*-methyl-D-glucose remains much lower and does not equilibrate even 30 min after incubation in α-cells. However, it has been difficult to evaluate the relationship between the glucose uptake activity and the glucose responsiveness of the cells in real time at the singe cell level. We show here that single pancreatic β-cells in which $[Ca^{2+}]_i$ increases in response to a high concentration of glucose have high 2-NBDG uptake activity. Not all of the β-cells with significant uptake of 2-NBDG exhibited an increase in $[Ca^{2+}]_i$ in response to glucose, however. These results suggest that not only glucose uptake but also the subsequent glucose metabolism plays a pivotal role in glucose-induced insulin secretion from β-cells (27). This is supported by the finding of the glucose-induced increase in $[Ca^{2+}]_i$ that is inhibited reversibly by the metabolic inhibitor DNP in β-cells, even though these cells show significant uptake of 2-NBDG. Because DNP had no effect on the 2-NBDG uptake into β-cells, it is suggested that glucose metabolism has little effect on the rapid equilibration of glucose uptake in β-cells. On the other hand, a small population of the islet cells showed no similar significant, rapid incorporation of 2-NBDG. All of these cells also lacked glucose-induced $[Ca^{2+}]_i$ responsiveness. These cells, therefore, were non-β-cells or possibly α-cells; although they eventually showed 2-NBDG uptake after longer incubation, their fluorescence intensities were much weaker than those of β-cells. These results show that 2-NBDG is an effective tracer of glucose transport activity in

pancreatic β-cells. 2-NBDG should also be helpful in clarifying the mechanisms underlying dynamic glucose uptake-function coupling in other glucose-responsive tissues and cells.

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Measurement of Glucose Uptake and Intracellular Calcium Concentration in Single, Living Pancreatic β-Cells

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