Fine Structure and Secretory Function of Islet β-cells in the Rat Pancreas Studied by Video Microscopy

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SUMMARY

A spatial organization of islet β-cells, a secretion of insulin, and a relationship between them were studied without cellular dissociation by using a video-enhanced contrast differential interference contrast (VEC-DIC) microscope. In the rat pancreas, endocrine islet cells appeared with less contrast than exocrine acinar cells, and contained secretory granules (c.a. 0.5 - 1.2 μm in diameter) more scattered than those in acinar cells. In individual islet cells, the exocytotic response (degranulation) appeared as abrupt light intensity changes of many secretory granules, when the concentration of glucose in the superfusion solution was raised from 1.5 mM to 15 mM. Such responses were observed in most islet cells simultaneously. By counting the number of responses in a unit of time, the time course was studied for quantitative estimation of the secretory activity. The glucose-induced exocytotic response was augmented in the presence of L-arginine (0.3 mM), and was suppressed completely by removal of Ca^{2+} from the medium. The glucose-sensitive cells were sensitive also to elevation of K^+ concentration in the medium from 5 mM to 65 mM, and to addition of A23187 (a Ca^{2+} ionophore, 1 μM), tolbutamide (a sulfonylurea, 100 μM) and bethanechol (a muscarinic agonist, 100 μM) to the medium. The cells that responded to glucose were immunopositive when fixed in mid-response and processed with an HRP-conjugated anti-insulin antibody. After a glucose-induced degranulating response, release of insulin to the extracellular medium was detected by the reverse hemolytic plaque assay. These results indicate that the exocytotic responses induced by glucose stimulation reflect the secretory activity of insulin-containing β-cells.

A three dimensional configuration of β-cells in Langerhans islet was best elucidated when cells were completely degranulated by extensive glucose stimulation. Several β-cells adhered each other, and formed many elliptically shaped clusters (40.3 ± 0.8 μm for long

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axis and 35.1 ± 0.5 µm for short one, n=56). The cluster of β-cells was clearly visualized also by confocal laser microscopy in tissues stained with lucifer yellow or 8-hydroxy-pyrene-1, 3, 6-trisulfonic acid trisodium salt (an anionic fluorescence chromophore) and by light microscopy in tissues immunostained with anti-insulin antibody. The clustered structure was also recognized in the normally circulated pancreas. The clusters were classified roughly into two classes by their morphological characteristics: a small-sized one consisting of 8.3 ± 0.1 cells (n=43) and a large-sized one consisting of 15.5 ± 0.5 cells (n= 13). The former were located in the center of islet, and the latter in the periphery.

In order to develop a method for analysing secretion of insulin dynamically, the secretory activity of β-cell cluster was examined quantitatively. Almost all β-cells in a single cluster showed patterns of exocytotic responses similar to each other upon glucose stimulation. Different clusters, sometimes even next neighbors, in an islet showed heterogeneous response patterns. This heterogeneity of β-cell clusters was further demonstrated by immunostaining of tissues fixed in the peak of degranulating response. The heterogeneity became less prominent when the clusters were stimulated with K^+-rich solution or A23187-containing solution. The patterns of glucose-induced exocytotic response at 35 °C were classified roughly into two types: one showing a rapid and transient response, and the other showing a delayed and persistent response. An ensemble average of the responses observed in 40 clusters revealed a biphasic pattern involving an early phase and a late phase, consistent with the earlier observation. These suggest that the biphasic secretion of insulin was ascribed to heterogeneous activities of different β-cell clusters. The time course of the degranulation pattern depended significantly on the size of cluster: the early phase was mostly found in the small-sized clusters, and the late phase in the large-sized ones.

In Langerhans islet, β-cells were arranged in many clusters. These clusters were subdivided into two groups by their cell population and by their degranulation pattern. We concluded that the small-sized cluster of β-cells are mostly responsible for the secretion of insulin during brief stimulation, whereas the large-sized cluster of β-cells may significantly contribute to the total insulin release only after prolonged stimulation. The cluster of β-cells functions as a physiological unit for insulin secretion, and a diversity of individual clusters of β-cells may be an essential basis for a multifactorial control of glucose homeostasis.

INTRODUCTION

Glucose homeostasis is maintained by pancreatic β-cells which secrete insulin in response to the glucose level of the blood. Studying detailed mechanisms of the release of insulin from secretory granules in the pancreatic β-cell is important for understanding disorders of insulin secretion. The amount of insulin released from the pancreatic tissue to the plasma or incubation media was first quantified biochemically and immunologically (Hales and Randle, 1963). At a single cell level, the degree of insulin secretion was estimated by the reverse haemolytic plaque assay (Salomon and Meda, 1986). This method is highly specific and sensitive to insulin, but is not rapid enough to resolve secretory responses in time-domain. Morphological examination of fixed preparations showed that exocytosis is the major mode for the regulated secretion in the β-cell (Orci, 1974). For analysis of the exocytosis in the time domain, the membrane capacitance (Ämmälä et al., 1993) and serotonin-oxydizing current (Smith et al.,1994) were measured electrophysio-logically in isolated single β-cells. However, no dynamic analysis of insulin secretion has been described in terms of exocytosis in the pancreatic tissue.

In sections from rodent pancreas, the β-cells comprise the central area of the islet structure surrounded by one or two layers of other endocrine cells (Orci & Unger, 1975). Most studies on islet β-cells implicitly assumed that all the insulin-containing cells in a preparation were functionally identical. Nevertheless, the β-cells were found to differ in many aspects. The difference was exhibited as for their individual sensitivity to glucose, secretory activity (Salomon & Meda, 1986; Van Schravendijk et al., 1992), protein synthetic activity (Schuit et al., 1988), electrical activity (Dean & Matthews, 1970), and metabolic redox responsiveness (Pralong et al., 1990; Heimberg et al., 1993) in vitro. There are only a few pieces of evidence that the β-cells are functionally different in vivo, and even

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less evidence that they are structurally different in the pancreatic tissue. It has not been clarified also whether there is some correlation between morphological characteristics and physiological heterogeneity in β-cells. Studies on such a structure-function relationship *in situ* have been highly difficult, where individual β-cells can differ in their different cellular and hormonal environments (Pipeleers, 1987).

Recently, the exocytotic response of a single secretory granule was directly visualized in the endocrine and the exocrine cells by using a video-enhanced differential interference contrast (VEC-DIC) microscope (Terakawa, 1989). The VEC-DIC microscopy has several advantages over other bright-field microscopies: 1) the resolution easily reaches the theoretical limit, 2) the image is a projection of optically sliced section, 3) the contrast of a living tissue is formed without any staining. This technique provided us with a means to study secretory activities of individual cells without dissociating them, thus in their normal morphological and physiological environments.

I used this high-resolution optics to visualize the spatial arrangement and the insulin-releasing activity of β-cells directly and simultaneously in the pancreatic tissue. Amplification of the video signal made it possible to visualize a small change in refractive index associated with an exocytotic response of a single secretory granule. The same video technique also made it possible to identify molecules contained in the granules when combined with immunological assays. Upon glucose stimulation, insulin was released from the tissue by the exocytotic response or degranulation. When occurred massively, the degranulation further improved optical conditions of the tissue and helped to reveal its morphological characteristics in detail. This led us to a new view of β-cell configuration: a clustered arrangement. Here, I describe dynamic properties of exocytosis for insulin release and their relationship with the clustered structure of β-cells.

MATERIALS AND METHODS

Pancreatic tissue preparation for microscopy

The caudal part of the pancreas (0.2 - 0.5 g) was excised from ether-anaesthetized rats (male Wistar, 200 - 300 g). The tissue was sliced in the middle of Langerhans islet, and immediately cut into small pieces, approximately 2.0 x 2.0 x 0.2 mm^3, using a razor blade under a binocular microscope. A small piece of the tissue was placed on a coverslip (18 x 18 mm, 0.17 mm thick), and held by placing another piece of coverslip on top of it as shown in Fig. 1. Two parallel ridges of petroleum jelly aside the tissue served as spacer between coverslips. The tissue was superfused continuously with the standard medium (see below) by passing it through the narrow space (volume = 13 x 13 x 3 mm^3) between the two coverslips. After 20 min of superfusion with the standard medium at 35°C, the concentration of glucose was raised from 1.5 mM to 15 mM for stimulation. A peristaltic pump (SJ-1220, Atto, Tokyo) was used for supplying media for superfusion. The temperature of the medium was adjusted by changing the voltage applied to a heater attached to an oil-immersion objective lens. For *in situ* observation, the rat was anaesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg). The abdomen was incised, and splenic veins were ligated and sectioned in order to detach the distal half of the pancreas from the peritoneum. The exposed tissue was covered with a thin sheet of wrapping foil except for a small region in the corpus of pancreas. A thin part of the corpus was observed by video microscopy.

Microscopy

The video microscopy was similar to that described previously (Terakawa, 1989). Pancreatic cells were observed under an inverted Nomarski microscope (Axivert 35, Zeiss, Obercochen) equipped with a x100 differential interference contrast (DIC) oil immersion

lens and a x2.5 insertion lens. The Nomarski microscope produced an image of the tissue optically sectioned to a slice of about 1 μm thick. The optical image was detected using a charge coupled device (CCD) camera (SSC-M350, Sony, Tokyo), and the contrast of the video image was enhanced (VEC) with a high-speed digital image processor (PIP-4000, ADS, Osaka). The enhancement was equivalent to amplification of the video signal by a factor of 4 - 5. The processed image was displayed on a video monitor screen (VM-1221, B/W, Hitachi, Tokyo) at a final magnification of x 4,000 or x 10,000, and recorded simultaneously using an S-VHS format recorder (AG-7750, Panasonic, Osaka). Pictures were reproduced from videotape by digitizing necessary frames and directly hardcopying them with a photographic printer (UP-D7000, Sony, Tokyo).

The confocal microscopy was carried out using an inverted microscope (TMD-300, Nikon, Japan) equipped with a confocal laser scanning system (MRC-600, Bio-Rad House, Hemel Hempstead, Hertfordshire, UK). Lucifer yellow (1 mM), lucifer yellow-conjugated dextran (100 μM), or 8-hydroxypyrene-1, 3, 6-trisulfonic acid trisodium salt (HPTS, 10 μM) was added to the superfusion medium and observed under epifluorescence illumination with a 488 nm argon-laser beam scanned over the specimen.
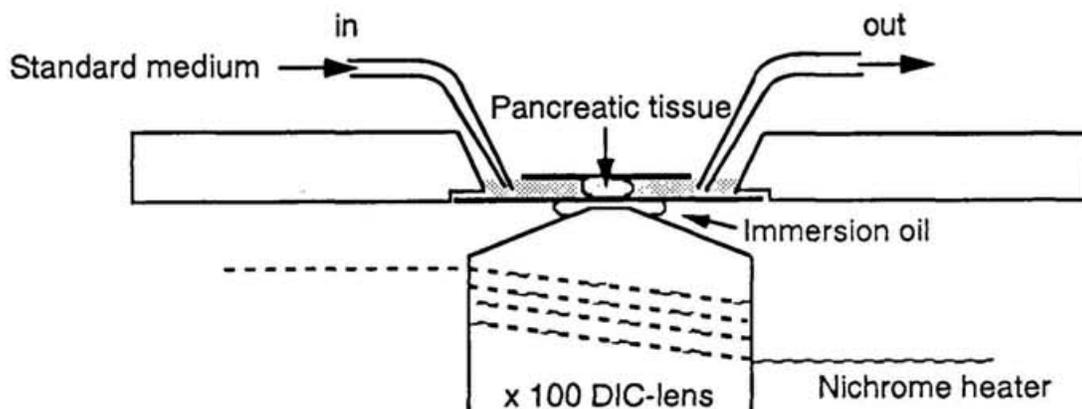

Fig. 1 Side view of the preparation and the microscopic apparatus

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Analysis of light intensity

To study the details of the exocytotic response, we analyzed the light intensity change of a single granule that underwent exocytosis. Release of highly condensed substances from the granule was assumed to give rise to a change in the refractive index, which would appear as a light intensity change under a DIC microscope. Sequential video images were stored in multi-frame memories of the digital image processor at an interval of 33 ms. The light intensity was then calculated by summing digital values of pixels in a square cursor set on the appropriate part of a secretory granule. This enabled us to measure the changes in light intensity for 2 - 4 s.

Frequency of exocytosis

Accurate numbers and timing of responding granules were analyzed after a real-time image processing for time-differentiation of the video record using an image processor (sequential subtraction mode in Argus-20, Hamamatsu Photonics). Only an abrupt change in real image formed a contrast transiently on the video monitor, and thus it was taken as the exocytotic event (Terakawa et al., 1991). The frequency of exocytotic events in a unit of time was counted for quantitative estimation of secretory activity in each islet cell.

Tissue fixation and immunostaining

To prove the presence of insulin-containing granules in the cells responding to glucose, we stained them immunochemically with an anti-insulin antibody (Sigma, St. Louis). We employed a streptavidin-biotin complex (ABC) method with 3-amino-9-ethylcarbazole (AEC) as a dye using a Histofine SAB-PO(R) kit (Nichirei). All procedures were performed at 4 °C. All media were prepared using a phosphate-buffered solution (PBS). We stimulated β-cells in the sliced pancreatic tissue by superfusion with 15 mM glucose-containing solution, and observed degranulation for 10 - 20 min. Then the pancreat-

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ic tissuewas fixed with 4% paraformaldehyde for 3 h immediately after video microscopic observation. After washing with PBS for 6 h, the tissue was immersed in 0.5% Triton-X100 for 72 h. The endogeneous peroxidase activity was blocked by overnight treatment of the tissue with 0.75% hydrogen peroxide. Nonspecific antigens were blocked by exposure of the tissue to 10% non-immune goat serum for 12 h. The conditioned tissue was then incubated in the medium containing the anti-insulin antibody (150 µg/ml) for 24 h. The excess free primary antibody was removed by washing with PBS, and the bound antibody was detected by sequential incubation with a medium containing biotinylated guinea pig anti-mouse IgG antibody (50 µg/ml) for 24 h, and then with a medium containing streptavidin-horseradish peroxidase complex (3 µg/ml) for 24 h. The peroxidase reaction was carried out in a mixture of 0.006% AEC and 0.02% hydrogen peroxide for a minute or less. The tissue was then washed with distilled water and observed under the same microscope in a bright-field mode. The tissue processed without exposure to the primary antibody was used as a negative control.

Detection of insulin secretion

Insulin release from islet β-cells was visualized by using a method modified from the reverse hemolytic plaque assay as described by Salomon & Meda (1986). Briefly, an anti-insulin antibody was conjugated to protein A-coated guinea pig red blood cells. The sliced pancreatic tissue (see above) was placed over a thin layer of the treated blood cells, and superfused first with the standard medium, then with a 15 mM glucose-containing medium. About 30 min after glucose stimulation, the medium for superfusion was replaced with the one containing a guinea pig complement. Insulin release was revealed by the appearance of hemolytic plaques which resulted from the complement-mediated lysis of red blood cells bearing insulin-anti-insulin complexes bound to protein A. The formation of plaques was observed with VEC-DIC microscope.

Solution and reagents

The standard medium used for all experiments contained (in mM): NaCl, 115; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1.2; KH_2PO_4, 1 ; $NaHCO_3$, 20; glucose, 1.5; HEPES, 16 (pH adjusted with NaOH to 7.35). Most chemicals were purchased from Wako (Osaka). Lucifer yellow dilithium salt and HPTS were obtained from Molecular Probes (Eugine, OR), bethanechol chloride and protein A obtained from Sigma Chemical (St. Louis, MO), and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) obtained from Dojindo (Kumamoto). All chemicals were dissolved into the standard medium immediately before use.

Data analysis

Data are presented as mean ± S.E.M. Statistical analysis was performed using Student's t-test. Significance was attributed at $p < 0.05$. All data were calculated and analyzed with a personal computer (Power Macintosh 8100/80, Apple Computer Inc., Dallas) using an application software (StatView4.0, Abacus Concepts, Inc. Berkeley, California).

RESULTS

I. Iinsulin secretion from pancreatic ß-cells

VEC-DIC view of pancreatic cells

Endocrine islet cells in a sliced rat pancreas were distinguishable from exocrine acinar cells by their different appearances under the VEC-DIC microscope (Fig. 2). The former formed compact masses which gave an impression of a flat shape. These cells showed a nucleus (c.a. 5 μm in diameter) polarized to some extent in the periphery of the mass. In contrast, acinar cells appeared in rounder shape. In each acinus, they gave an impression of petals of a flower. They showed a very vague nucleus but a clear polarity, with a group of zymogen granules located toward the center of the acinus. Occasionally, a lumen was visible between them. The borders between islet cells were less clear than those between acinar cells. All the granules in the islet cells were scattered as dark or bright spots 0.5 - 1.2 μm in diameter. A layered structure of the mantle and core in a single granule, as a characteristic of insulin-containing granules observed by transmission electron microscopy (Lacy, 1961), could not be resolved. Most of the secretory granules in islet cells were moving very slowly (< 1.0 μm/min) in various directions. No gliding movement, as described in cultured endocrine cells (Lacy *et al.,* 1975; Somers *et al.,* 1979), was noticed under the present condition.

Glucose-induced single exocytotic response

All of the secretory granules were very quiescent when the tissue was superfused continuously with a control solution containing 1.5 mM glucose. However, when the concentration of glucose was raised from 1.5 mM to 15 mM, the granules abruptly changed in appearance and brightness, and disappeared one after another (Fig. 3). Neither a rapid movement of granules toward the membrane nor rapid swelling was noticed before the

abrupt response. In a single granule, this response was completed in a period of 200 ms. After such an abrupt response, the image of the same granule was no longer restored by changing the focus. Such a quantal response in the cell induced by glucose stimulation was rapid, usually showing the first granule change within 30 s after raising the glucose concentration, and was random, taking place without any obvious preferential site. We hypothesized that these quantal responses of granules were the exocytosis or degranulation of insulin-containing granules in β-cells. We could not directly detect insulin in intercellular spaces diffusing to blood capillaries. A few islet cells which did not respond to elevation of the glucose concentration were further tested for their glucose sensitivity, by superfusing first with 5.5 mM glucose-containing medium for 20 min and then with the 1.5 mM glucose-containing medium. Upon reduction of the glucose concentration from 15 mM to 5.5 mM, no response was observed. However, upon reduction from 5.5 mM to 1.5 mM, a significant rate of degranulation was observed in these cells (data not shown).

Light intensity and time-differential images of exocytotic response

A dynamic aspect of the single quantal response was studied by analyzing the change in light intensity of a granule associated with the change in image of the granule. Most granules appeared bright when observed in underfocus and dark when observed in overfocus, as their refractive indices were higher than their surroundings. Some granules tightly surrounded by other granules appeared grey and were barely distinguishable. In the islet cells stimulated by 15 mM glucose, many granules showed an abrupt change in light intensity with a time course of 100 - 200 ms (Fig. 4A). In all the granules that responded, the change in light intensity was always monophasic. However, the sign of the change was bidirectional. From a baseline, the light intensity either decreased or increased depending on the absolute value of initial light intensity. Nevertheless, the direction consistently indicated that the refractive index decreased in these granules. The baseline of the light

intensity curve recorded before the abrupt change was quite stable. After the rapid deflection, the curve returned to the original level slowly and slightly.

After the time-differential processing of recorded images, a rapidly changing part could be exclusively displayed against a uniformly grey background. Therefore, the image of a granule during its rapid light intensity change could be clearly detected by converting recorded images into time-differential images (Fig. 4C). The clarity of the differential images increased as the rate of light intensity change of a granule increased. Occasionally, an edge of the responding granule in the differential image was trimmed with a strong contrast, so that the clarity of the image further increased.

Immunopositivity to anti-insulin antibody

We stimulated islet cells in sliced pancreatic tissues by superfusion with 15 mM glucose-containing solution, and observed degranulation to some extent. Then we fixed the tissue and stained it using anti-insulin antibody. After several days of staining, the same field was brought under the microscope for examination. The staining was dense in the islet cells sensitive to glucose (Fig. 5A). In each cell, staining was found in individual granules. The islet cells fixed after complete degranulation exhibited no staining either in the cytoplasm or in the nucleus (Fig. 5B). When the primary antibody was omitted as a negative control, no secretory granule was stained in any islet cells (Fig. 5C). It was difficult, though, to assess the immunopositivity in the whole region of the islet tissue, as the antigen was detectable at a depth less than 30 μm from the tissue surface under our experimental condition. The acinar cells showed no staining under any condition (Fig. 5D).

Insulin secretion detected by hemolytic plaque assay

The pancreatic tissue was placed over a thin layer of protein A-coated red blood cells sensitized with the anti-insulin antibody, and stimulated with 15 mM glucose. After

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observation of exocytotic responses in the islet cells, the field of view was changed to the area downstream to the pancreatic tissue to observe red blood cells. As shown in Fig. 6E, the cells became gradually brighter and swollen to form ghosts. This hemolysis was observed only when the tissue was stimulated with glucose. The complement-mediated lysis was completed in 2 to 3 min in a single erythrocyte. In contrast, the erythrocytes located upstream to the pancreatic tissue never showed the lysis. Hemolytic plaques were not detected, when any component of the assay was omitted, i.e., when red blood cells were not coated with protein-A before antibody application, when a fresh serum of guinea pig was used as a substitute for the anti-insulin serum, when the addition of complement was omitted, or when the exocytotic responses of islet cells were not observed by unknown reason. The plaque formation was clearly correlated with the exocytotic responses in the islet, although the quantitative relationship between the number of exocytotic responses and the degree of plaque formation could not be determined.

Secretory activity measured as the frequency of exocytosis

By counting the number of quantal responses in a unit of time (mostly 30 s), we studied the time courses of secretory activity in many islet cells. As the central area of an islet is known to be occupied by insulin-containing β-cells (Orci and Unger, 1975), we mostly observed cells in that area. Since the frequency of responses in individual cells was very low, we counted it in a fixed area (100 μm^2) of the observation field (usually containing 5 - 6 cells). Typically, upon continuous stimulation, the major response started about 30 s after elevation of the glucose concentration, reached a peak in 2 to 3 min, and then subsided in 5 to 10 min (Fig. 7A). Similar responses to glucose were observed in 32 preparations. Brief (10 min) stimulation with glucose induced a response similar to that induced by the longer one (20 min). However, when the same brief stimulation was applied

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repetitively three to four times, the response was gradually augmented (Fig. 7B). The same effect was observed in four preparations examined consecutively. The glucose-induced response was augmented in the presence of 0.3 mM L-arginine (Fig. 7C), and was suppressed by removal of extracellular Ca^{2+} by addition of 2 mM ethylene glycol-bis (β-aminoethyl ether) N, N, N', N'-tetraacetic acid, EGTA, to the medium (Fig. 7D).

A secretory activity was induced in glucose-sensitive cells (n=4) by elevation of the K^+ concentration in the medium from 5 mM to 65 mM in the presence of 1.5 mM glucose (Fig. 8A). The quantal responses were also induced by the application of reagents known to release insulin. Tolbutamide (a sulfonylurea, 100 μM) and A23187 (a Ca^{2+} ionophore, 1 μM), each examined in four preparations, rapidly induced responses in the presence of 1.5 mM glucose (Fig. 8B and C). The responses induced by these chemicals tended to continue even after removal of the reagents.

Fig. 2. Video-enhanced contrast differential interference contrast image of a sliced pancreatic tissue

A lobule of pancreatic tissue was sliced to a small piece (about 2.0 x 2.0 x 0.2 mm³), and an edge of the piece was observed by video-enhanced contrast differential interference contrast (VEC-DIC) microscopy (thickness of the focal plane, 1-3 μm). The focus was placed at a depth of about 10 μm from the tissue surface. Islet cells are shown to the right of the line between the arrows. Many islet cells containing a nucleus (asterisk) and secretory granules are shown. An exocrine acinus consisting of several granule-rich well-polarized cells is shown to the left of the same line. Calibration bar, 5 μm. L, lumen of an acinus.
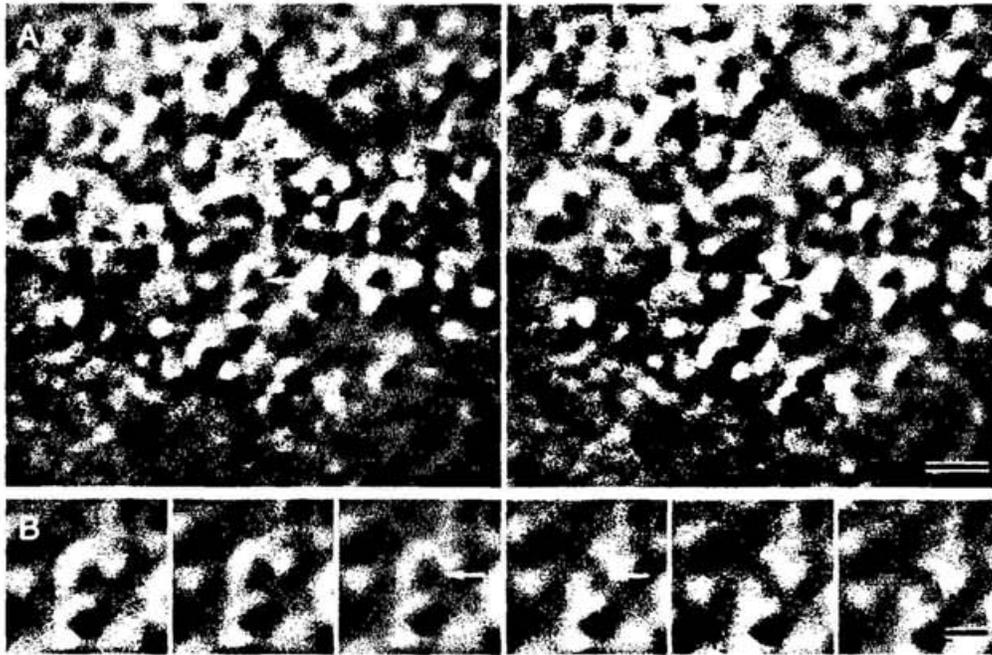
Fig. 3. Exocytotic response of a single granule in an islet cell induced by glucose stimulation

A, successive images enlarged from an islet cell shown in Fig. 2. Many secretory granules were observed in a single cell. A granule showed an abrupt change (arrows) 5 min after the concentration of glucose in the medium was raised from 1.5 mM to 15 mM. The interval between two frames was 66 ms. B, sequential video images showing an exocytotic change of a single granule (arrows) in a β-cell. Images were enlarged from the area containing a responding granule in A (arrow in A). The interval between each frame was 66 ms. Calibrations, 2 μm for A, and 1 μm for B.
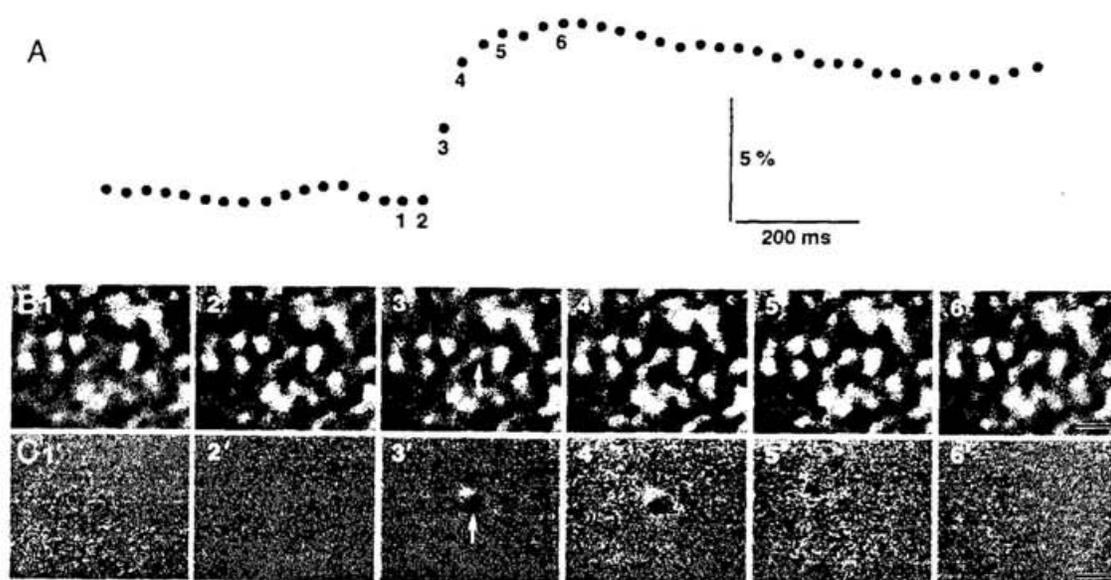
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Fig. 4. Changes in light intensity and in image of a single granule undergoing an exocytotic response

A, change in light intensity of a secretory granule measured from the sequential images shown in B. The amplitude is expressed as a percent of the initial light intensity in the region of the granule. Sequential video images were stored in multiple frame memories of a digital image processor, and then the light intensity was calculated as described in Methods. Numbers indicate temporal correspondence between the light intensity data and the image frames shown in B. Time resolution for data points are 33 ms. B, sequential images of β-granules showing a single exocytotic response (arrow). C, a sequence of video frames obtained by real-time image processing for time-differentiation, capturing an exocytotic response (arrow). C2 was a result of subtracting B2 from B1, C3 was a result of subtracting B3 from B2, and so on. C1 was made by subtracting B1 from its previous image (not shown). Calibrations, 1 μm.
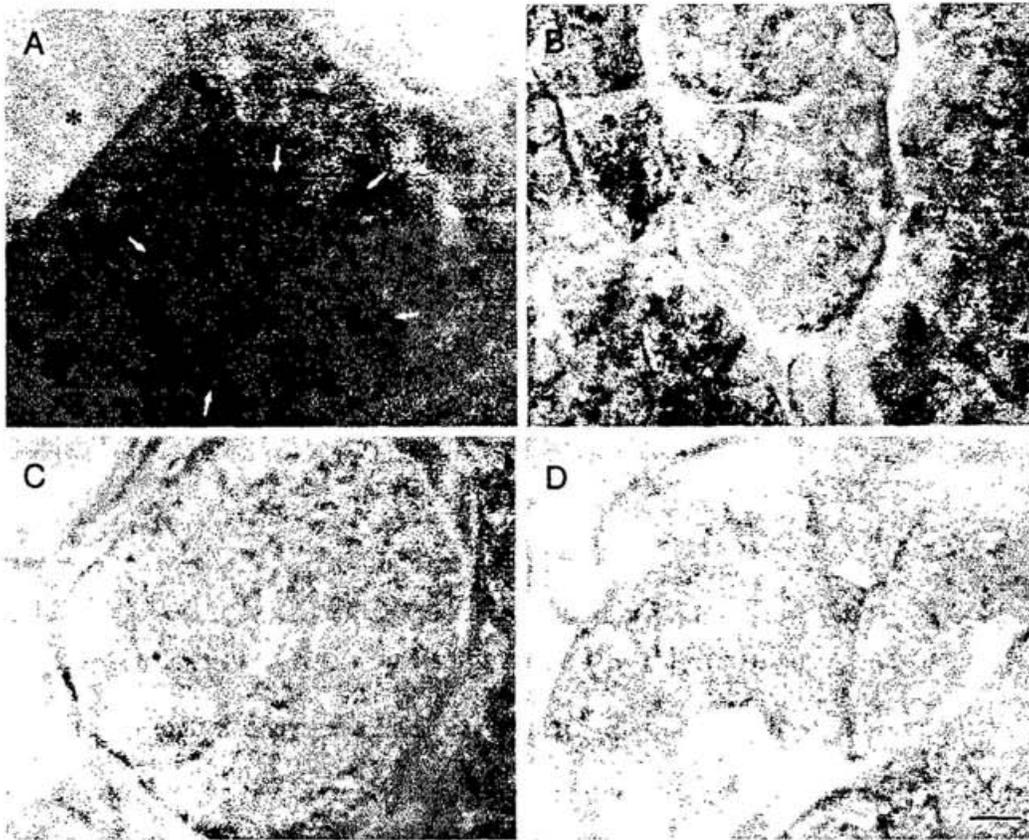
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Fig. 5. Bright-field images of pancreatic cells stained by the ABC-AEC method using an anti-insulin antibody

A, islet cells fixed after observing several exocytotic responses induced by glucose. Immunopositivity above a control level is shown as many dark spots (arrows) representing the insulin-containing secretory granules. Asterisk, islet cells which were unresponsive to glucose stimulation. **B**, islet cells fixed after complete degranulation by glucose stimulation. **C**, islet cells stained without using non-immune IgG (negative control). **D**, acinar cells processed for the same staining. Primary antibody binding was detected using a biotinylated secondary antibody and a streptavidin-peroxidase complex. Calibration, 5 μm.
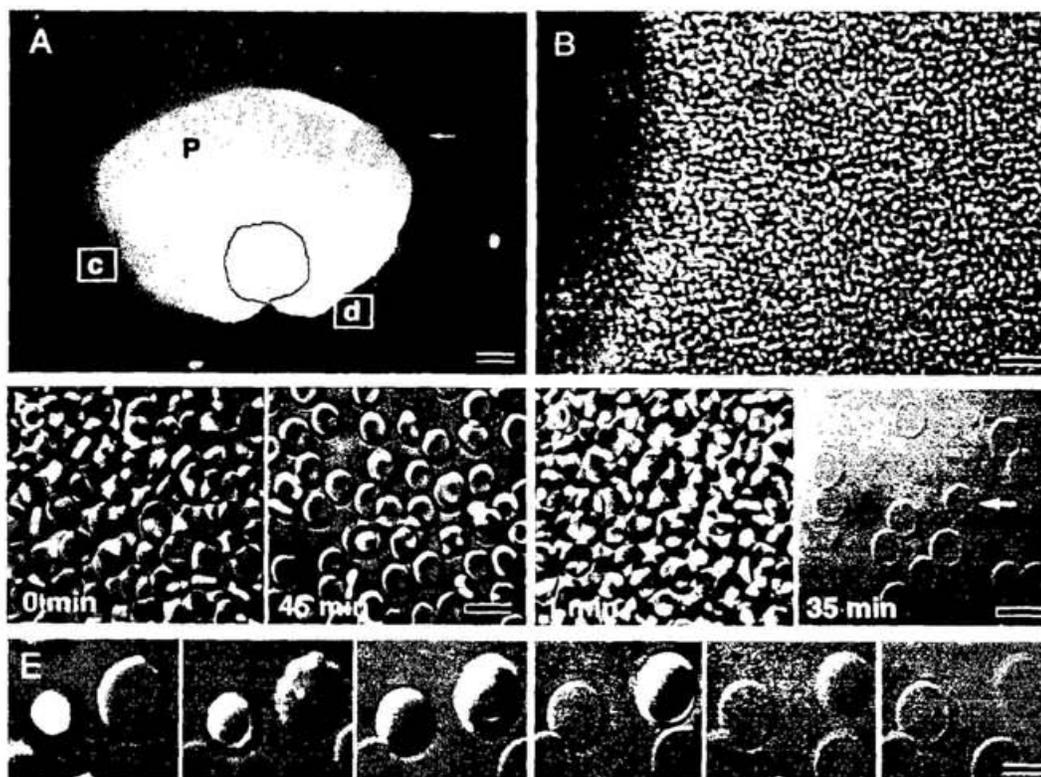
Fig. 6. Detection of insulin by the reverse hemolytic plaque assay

A, low magnification view of a pancreatic tissue (P) and sensitized red blood cells (in the area indicated by arrow, actually invisible) in the superfusion chamber. The medium flowed from left to right. A circle indicates the location of the islet. **B**, magnified image of red blood cells in region 2 in A. Formation of hemolytic plaques could not be visualized under this magnification. **C**, images of red blood cells in region c in A before and after stimulation with 15 mM glucose. Time after stimulation is indicated in each frame. Number of red blood cells remained in the chamber was decreased because the superfusion medium carried away some of the cells. **D**, images of red blood cells in region d in A. Hemolysis is easily identified by the faint image of erythrocyte ghosts (arrow). In this preparation, secretory granules in islet cells showed exocytotic response to glucose stimulation. No plaque was developed to the left of the tissue (region c) or when the assay was performed in the absence of complement. **E**, sequential images of single red blood cells during the lysis. The interval between each frame was 30 s. Images were obtained with a dissecting microscope (A) and a VEC-DIC microscope (B - D). Calibrations, 200 μm for A, 50 μm for B, 10 μm for C and D, and 5 μm for E.
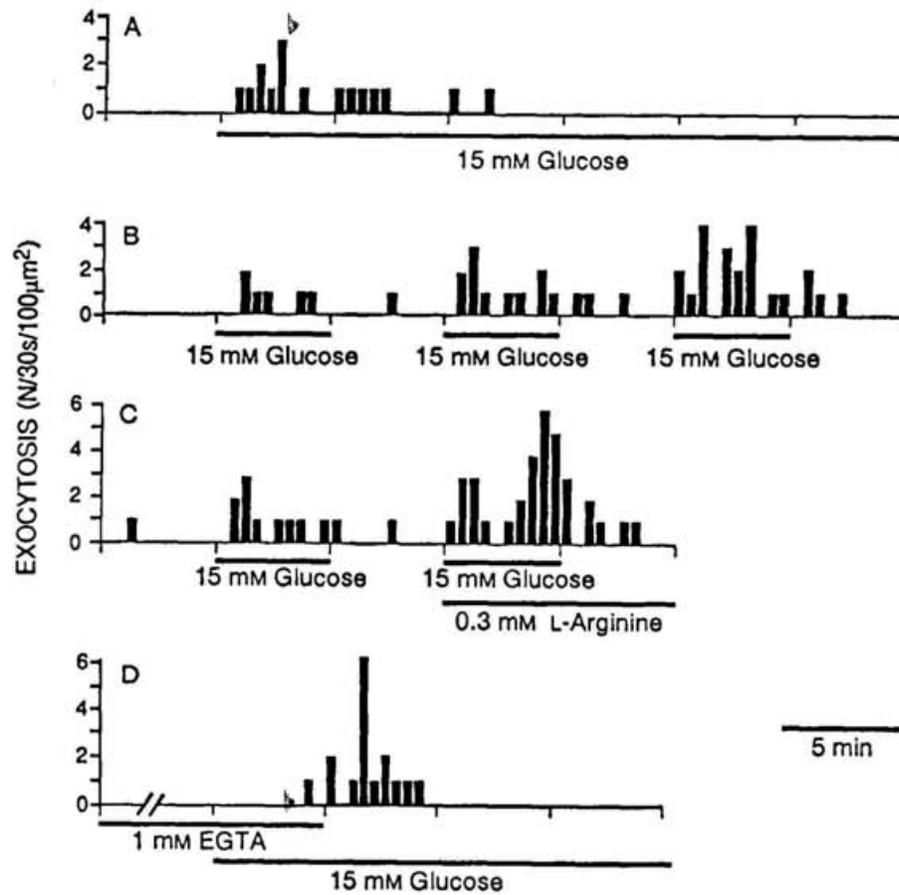
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Fig. 7. Typical frequency histograms of exocytotic responses in islet cells induced by glucose stimulation

A, continuous stimulation with glucose inducing a rapid onset and desensitization of the response. B, facilitatory effect of repetitive stimulation with glucose. C, augmentation of glucose-induced response by the presence of L-arginine. D, suppression of glucose-induced response by removal of Ca^{2+} from the medium. Records were obtained from different preparations. Number of quantal responses (exocytosis) in every 30-s period was counted in the center of islet in an observation field of 100 μm^2 and expressed as events (ordinate). The concentration of glucose in the medium was raised from 1.5 mM to 15 mM during the period indicated by horizontal bars.
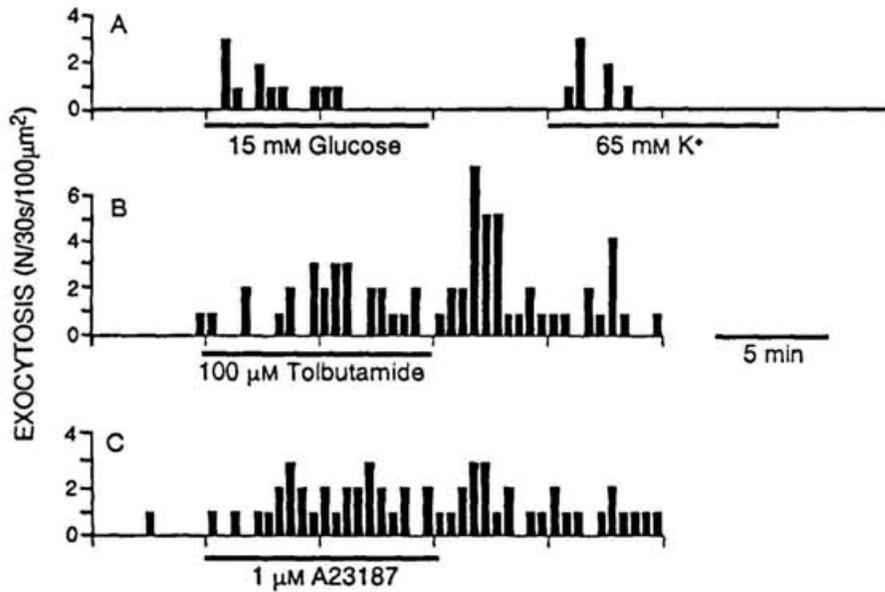
Fig. 8. Effects of insulin secretagogues on exocytotic responses in islet cells

Reagents were applied during periods indicated by horizonal bars. **A**, the concentration of K$^+$ was raised from 5 mM to 65 mM by substituting for Na$^+$ after stimulation with 15 mM glucose. The responses induced by both stimuli subsided within 10 min. **B**, effect of 100 μM tolbutamide. **C**, effect of 1 μM A23187. The responses induced by tolbutamide and A23187 easily continued even after removal of the stimulants. The ordinate is the same as described in the Fig. 7 legend.

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II. *Three dimensional configuration of ß-cells*

Clustering of islet cells in an excised pancreas

In a freshly sliced tissue of the rat pancreas observed under a VEC-DIC microscope, islet cells were not easily outlined individually but rather found in a form of clusters (Fig. 9). The clusters were somewhat similar to exocrine acini, although clearly distinguishable from them in several aspects (see, RESULTS I). Each cluster was elliptical in shape rather than spherical when observed with the focal plane adjusted in the center of the cluster. The lengths of long axis and short axis were 40.3 ± 0.8 and 35.1 ± 0.5 μm (n=56), respectively, and the eccentricity was 0.45 ± 0.01. One cluster contained 10.0 ± 0.4 (n=56) cells tightly adhered to each other. The border between these cells was not visible in fresh preparations. Secretory granules (0.5 - 1.2 μm) in each cell were concentrated near the center of the cluster. They were found to move extremely slowly (< 1 μm/min) by time-lapse video-recording. Similar images of the cluster were also observed in Langerhans islets of other rodents such as the mouse, rabbit, and guinea pig. Exocrine acini surrounding an islet appeared like flowers of 40 - 50 μm in diameter containing 6 - 8 cells resembling petals. A lumen was visible in the central region of the acinus. However, the border between an islet and surrounding acini was not always clear, because the shape of clusters at the edge of the islet sometimes very closely resembled that of acini (Fig. 12C).

Confocal images of clusters

The clustered units of the islet cells were more clearly visualized by staining the tissue with membrane-impermeable fluorescent dyes such as lucifer yellow, lucifer yellow conjugated dextran, and HPTS. Under a confocal laser microscope, the fluorescence intensity was particularly strong in spaces between clusters and in some capillaries, but weak between islet cells in each cluster (Fig. 10). Similar results were observed in 12 preparations mounted in various directions. The size of fluorescently distinguished clusters

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was about the same as that observed by the VEC-DIC microscopy. Nuclei and some population of granules in islet cells were also stained with lucifer yellow (1 mM) and HPTS (10 μM). It was difficult to recognize the same clustered units after dissociation of islets from the pancreatic tissue by the collagenase digestion method (data not shown). No acinar cell in the same preparation was stained with lucifer yellow. Intercellular spaces between acinar cells were usually also very dark. At this moment, the mechanism of staining intracellular organelles of the islet cells with lucifer yellow and HPTS is not clear.

Clustering of islet cells in a normally circulated pancreas

An individual pancreatic cell of a living rat *in situ* was blurred rhythmically at a high magnification, as the tissue was pulsating by circulation. Even so, the islet cells and their clustered structure could be recognized somehow in the region of capillary glomeruli (Fig. 11). Capillaries could be identified very easily by many erythrocytes circulating rapidly along them. Clusters of islet cells *in situ* showed the same characteristics as described above, although the difference between the islet cell clusters and the exocrine acini was less obvious. The nuclei of islet cells were only faintly visible and the secretory granules were more localized closer to the centre of the cluster. The granules of the same cells were moving very slowly (< 1 μm/min) as in a sliced tissue, and became quiescent with glucose stimulation. Forming several cranks, capillaries surrounded the individual clusters only partly along their two or three sides, but not completely around them. Capillaries did not penetrate through a single cluster in its center. The clusters were sometimes juxtaposed to other clusters at a faint border not demarked by the capillary, and thus appeared to be ordered as a cord-like structures. Such a border between clusters was more distinct than the one between individual cells in a cluster.

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Two classes of the clusters

Images of the deep part of an islet were usually blurred by a large thickness of the tissue (more than 100 μm) and a high density of secretory granules. Thus it was unclear whether clusters were formed in a whole islet. However, the images became clearer when the cells were degranulated by extensive glucose stimulation. In fact, superfusion of the tissue with 15 mM glucose-containing solution for 40 min brought about significant degranulation in many cells. After such degranulation, borders between clusters and between individual cells became wider and obvious in a whole part of the islet. Then, at least two classes of clusters were distinguishable with respect to their cell population. The number of cells in each cluster was countable by shifting the focus through the cluster. The size of a cluster determined by the number of cells was classified into two groups: small-sized (Fig. 12A) and large-sized (Fig. 12B). The small cluster consisted of 8.3 ± 0.1 (n= 43), and the large one of 15.5 ± 0.5 (n=13) cells. The lengths of long and short axes were 37.4 ± 0.46 and 33.7 ± 0.4 (n=43) for small-sized cluster, and 49.2 ± 1.3 and 39.8 ± 1.3 (n= 13) for large-sized one. The eccentricities of small-sized and large-sized clusters were 0.41 ± 0.01 and 0.57 ± 0.02, and were significantly different ($p < 0.01$). We could not determine the accurate ratio between the small and large groups in an islet. The ratio seemed to vary depending on the size of an islet, the region of pancreas, and the age of the rat. The locality of the clusters was examined by scanning the field of view. It appeared that there was a general organization of clusters in an islet: the small-sized cluster tended to be located near the centre of an islet (Fig. 12C-a), whereas the large-sized clusters were located in the periphery (Fig. 12C-b), although the both were not separated in distinctive zones as in a cortex-medulla structure. Cells insensitive to glucose were located at the edge of the islet and formed clusters as well.

Fig. 9. VEC-DIC image of a cluster of ß-cells

β-cells (center) appeared in a compact group partly surrounded by blood capillaries (arrowheads), and exhibited secretory granules slightly localized to the center of the cluster. Such a group or cluster was juxtaposed to other clusters without interrupted by capillalies (compare with Fig. 2). The border to a neighboring cluster is shown by arrows. *β*-cells were identified by observing the glucose-induced exocytotic response. The image was captured at the peak of degranulating response. Acinar cells (right side) appearing in "petals of flower", showed no glucose-induced exocytotic response. Calibration bar, 5 *μ*m.
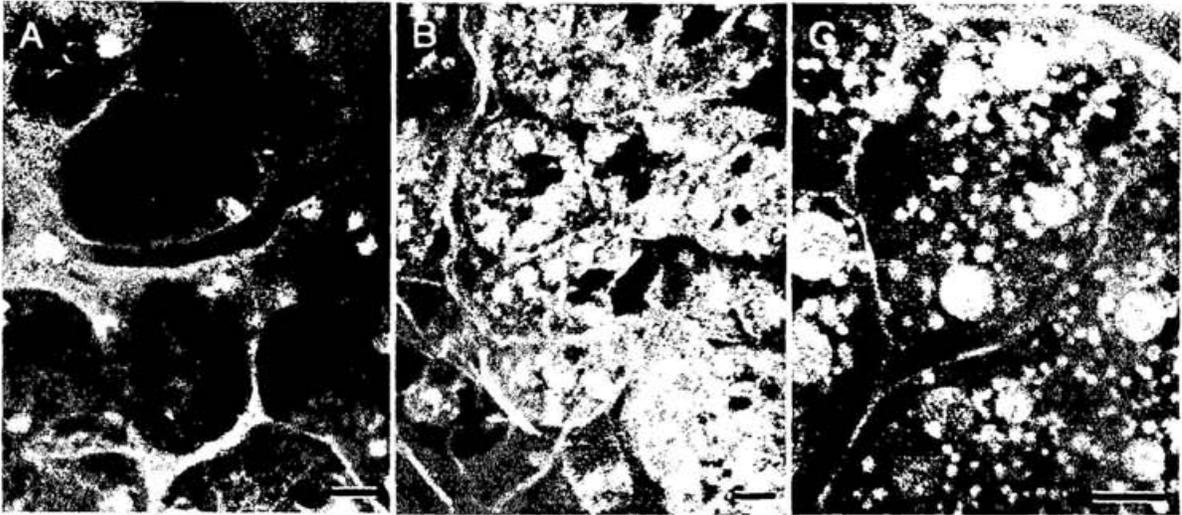
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Fig. 10. Fluorescence images of clusters obtained with a confocal laser microscope

Images of islet-cell clusters were obtained from sliced tissues of pancreas. The tissues were immersed in media containing lucifer yellow-labeled dextran (A), lucifer yellow (B), and HPTS (C). All of borders between clusters were high-lighted, representing the presence of interstitial spaces. In contrast, borders between individual cells in a cluster were dark. Nuclei and some population of granules in islet cells in B and C were also intensely stained with the dyes. The thickness of the focal plane, ~3 μm. Calibrations, 10 μm.
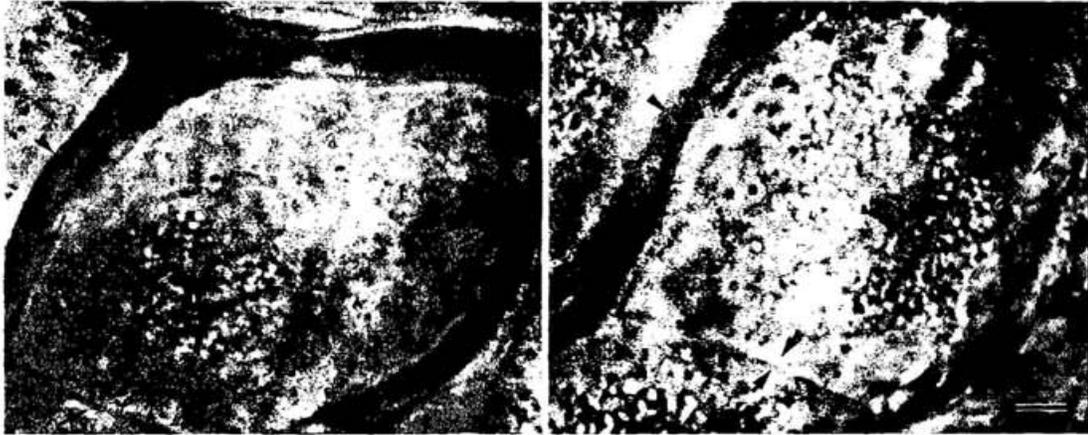
Fig. 11. Clusters of islet cells in normally circulated pancreas

The pancreas was partly detached from the peritoneum and flipped out of the rat abdominal cavity without interruption of the blood flow. VEC-DIC images (left and right) were obtained by changing the focus slightly ($\sim 3~\mu$m) in such a tissue. The clusters of islet cells *in situ* were partly surrounded by capillaries (arrowheads), and otherwise attached to each other with a barely discernible border (arrows). Observation of the exocytotic responses in these preparations were difficult because of many reasons including pulsation, flickering of erythrocytes, lower contrast, and so on. Calibration, 10 μm.
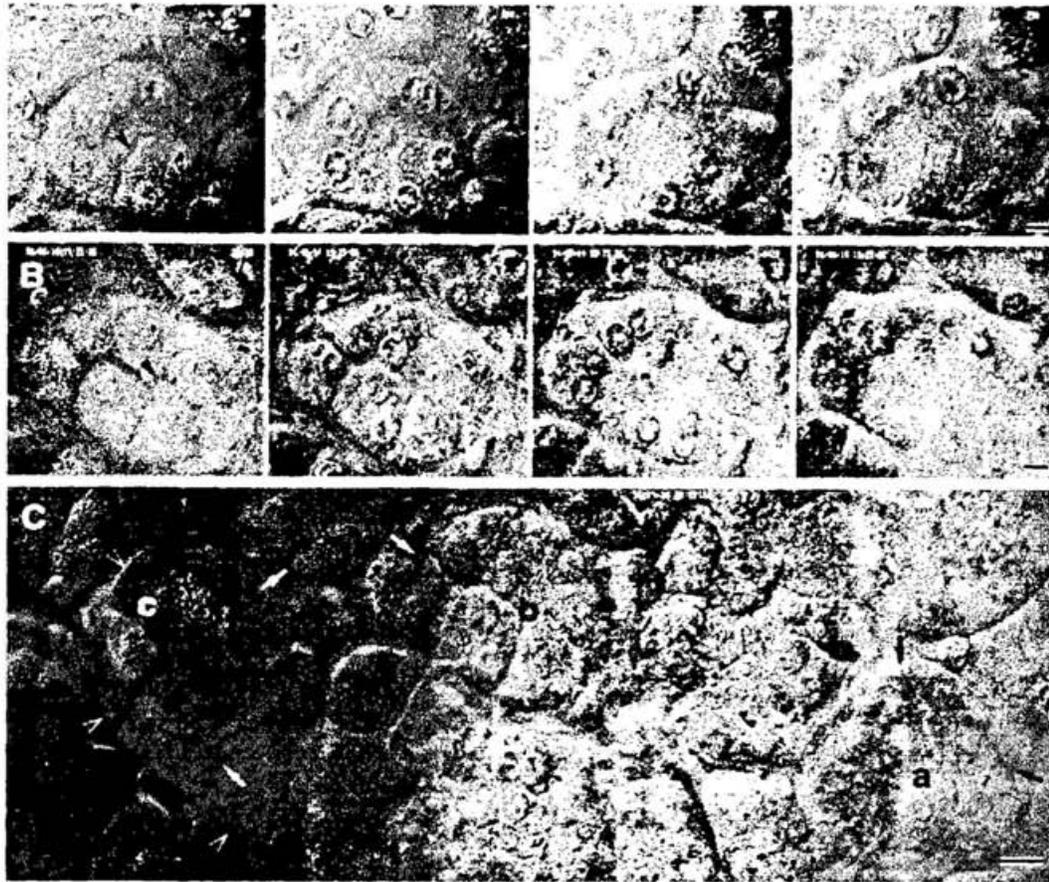
Fig. 12. Three dimensional configuration of β-cell clusters in a pancreatic tissue

Changing the focus revealed the number of β-cells in clusters: one cluster consisted of 8 cells (**A**; a in **C**) and the another of 16 cells (**B**; b in **C**). Spaces between β-cells (arrowheads) became clear when cells were degranulated by glucose stimulation. The β-cells were identified by immunostaining with an anti-insulin antibody as shown in Fig. 5. **C**, computer-reconstructed image of the optically sectioned islet. The number of β-cells in a cluster was correlated with the location of the cluster in an islet. The 8 cell-membered cluster (a in **C**) was located near the center of islet, and the 16 cell-membered cluster (b in **C**) was located in the periphery. All images were taken 60 min after glucose (15 mM) stimulation, and the number of secretory granules in the β-cells was considerably decreased in this preparation (compare with Fig. 9). Note a cluster insensitive to glucose (c in **C**) at the periphery of islet. The border between the islet and acini is shown by arrowheads. Calibrations, 5 μm in A and B; 10 μm in C.

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III. *Exocytotic response in ß-cell clusters*

Heterogeneity in clusters of *ß*-cells

By counting the number of the exocytotic response in a unit time (mostly 30 s), we studied the time course of secretory activity in many clusters. The frequency of degranulation events in a majority of the cluster was low (< 1 event/min). However, in about 20% of clusters, the frequency was distinctively high (> 10 event/min). When a response in a cluster continued at a high frequency, almost all secretory granules were depleted within 30 min (Fig. 13). Granules were not newly synthesized during 60 min of glucose stimulation in sliced tissues. After such a degranulating response, each cell shrank slightly, leaving a clear intercellular space. Therefore, it was possible to define the outline of the *ß*-cells and the number of *ß*-cells in the cluster (Fig. 14A). All cells in a single cluster responded to glucose stimulation with a similar time course (Fig. 14B, 1-8). However, different clusters, sometimes even next neighbours, responded with different time courses (Fig. 14B, 9). Some clusters responded in 30 min, and some others responded in 60 min. This heterogeneity of *ß*-cell clusters was further demonstrated by immunostaining of sliced tissues. The staining observed by VEC-bright field microscopy was exclusively in individual granules in the islet cells fixed before massive degranulation. In tissues fixed in the peak of degranulating response, immuno-positivities were different from cluster to cluster, depending on the number of secretory granules remaining (Fig. 15).

Two-phase pattern of degranulating response

The response pattern of each cluster was variable to a great extent (Fig. 13 and 15). Typically, three different response patterns were observed among clusters upon glucose stimulation (Fig. 16). In most clusters, the major response occurred transiently only in 2 to 3 min after glucose stimulation and then desensitized gradually in 5 to 10 min (type A). This initial or early transient type of response was observed in about 80% of clusters. In

some clusters, the same early response was followed by another large response which developed gradually and reached a peak at 30 to 40 min (type B). This combined type was observed in about 10% of clusters. In other clusters, a response occurred only in the late phase that started gradually at about 10 min after glucose stimulation (type C). This late type was observed in about 10% of clusters. An ensemble average of responses observed in 40 clusters at a temperature of 35°C revealed a biphasic pattern involving an early phase (0 - 7 min) and a late phase (7 - 30 min) as shown in Fig. 17. The late phase response was completely suppressed when the same observation was made at 25°C. The average frequency of the quantal responses at 35°C and at 25°C was 1.40 and 0.74 per min during the early phase, and 2.31 and 0.04 per min during the late phase, respectively. The total numbers of degranulation in the early and late phase were counted separately in individual clusters and compared with respect to the size of clusters. The number of degranulation in the late phase was significantly larger in the large clusters than in the small ones (Table 1).

Degranulation patterns and reagents known to induce insulin secretion

Clusters of β-cells showed more or less degranulation when examined in sliced pancreatic tissues superfused with medium containing bethanechol (BCh, 100 μM), a muscarinic agonist. The extent and time-course of degranulation in each cluster were variable (Fig. 18). One showed a weak response upon BCh stimulation compared with that induced by glucose stimulation, but later showed a much larger response. Another cluster showed a large response to BCh. The total numbers of degranulation observed in 5 min of BCh stimulation and in following 5 min of glucose stimulation were 8.12 ± 2.63 (n=8) and 10.5 ± 2.31 (n=8), respectively. When averaged in several preparations, no significant statistical difference was observed between these responses. However, individual clusters had clear heterogeneities in terms of their differential sensitivity to the muscarinic agonist and glucose. The patterns of exocytotic responses were also examined by the application of

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reagents which are known to elevate the concentration of intracellular Ca^{2+} in β-cells. Elevation of K^+ concentration in the medium from 5 mM to 65 mM induced a response similar to type A. A23187, a Ca^{2+} ionophore, induced responses similar to type C at a concentration of 1 μM. In both cases, most clusters homogeneously showed a similar time course.
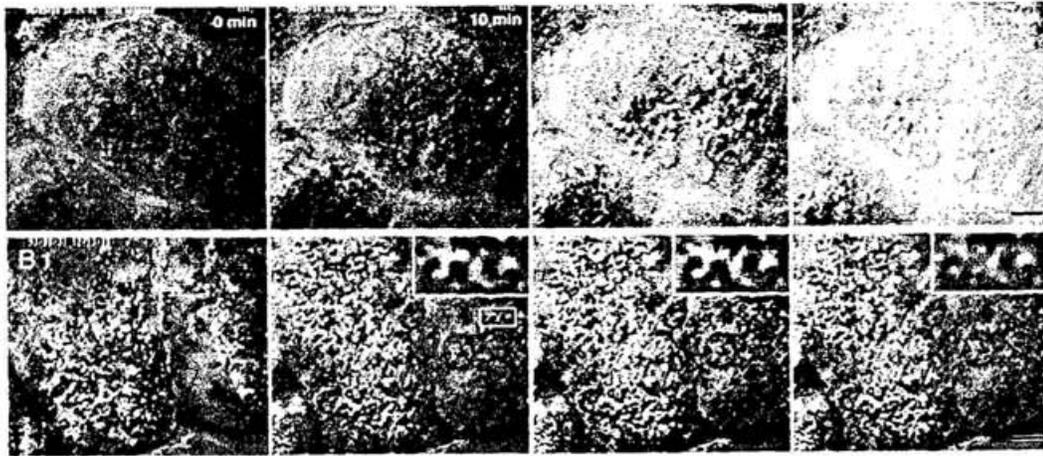
Fig. 13. Glucose-induced degranulation in the clusters of ß-cells

A, tissue was superfused with a medium containing 15 mM glucose for the time indicated. Cells in the central cluster showed response patterns similar to each other, but those in the cluster of the lower left corner responded with a different time course. The brightness in the cluster increased as the number of secretory granules decreased. **B**, images of a tissue taken before stimulation (1) and 40 min (2-4) after the onset of glucose stimulation. The cluster to the right side showed a late phase response and degranulated completely in 40 min, while the one to the left showed an early phase only. Insets in B (2-4) show the framed area at a high magnification, in which a secretory granule (arrowhead) disappeared. The interval between each frame was 66 ms. Calibrations, 5 μm.
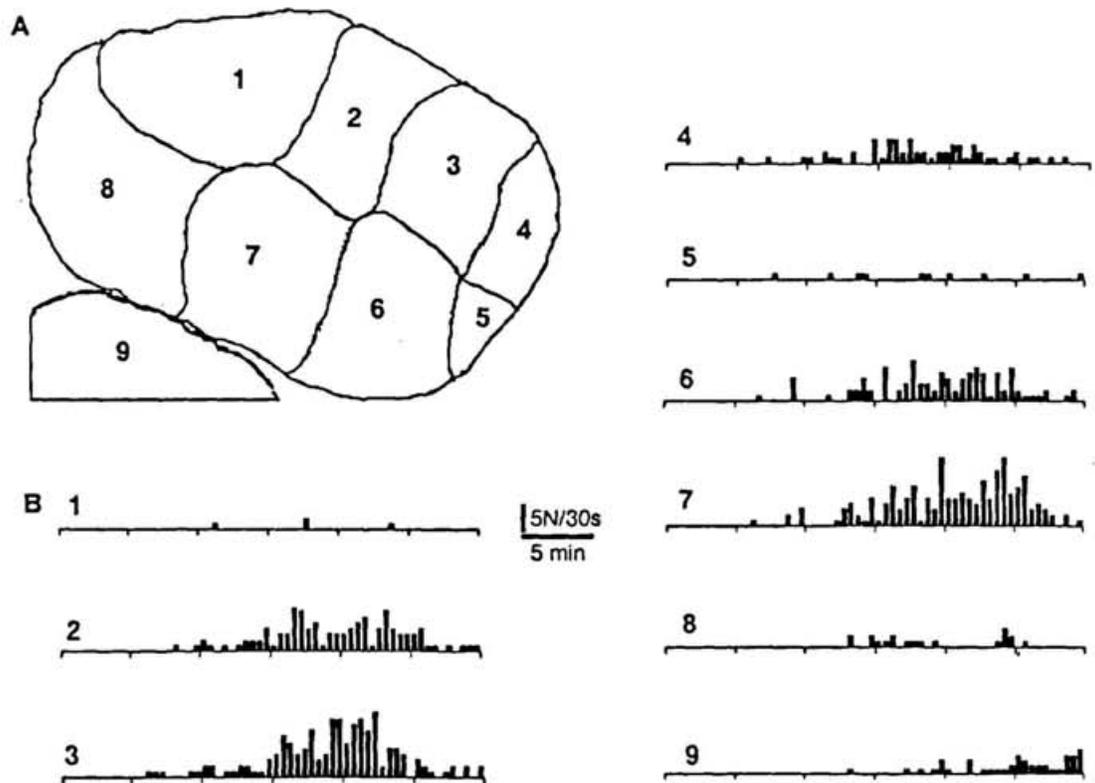
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Fig. 14. Homogeneity of response patterns in individual ß-cells in a cluster

A, schematic representation of the outline of ß-cells in a cluster shown in Fig. 12B. Numbers indicate each ß-cell. B, frequency histograms of exocytotic response in each ß-cell shown in A. The number of quantal response (N) in every 30 s period was counted and expressed as the height of bars. The concentration of glucose in the medium was raised from 1.5 mM to 15 mM at the beginning of each histogram. Cells (1) - (8) in the same cluster responded with similar time courses, whereas a cell (9) in another cluster responded in a different pattern with a delayed mean response time.
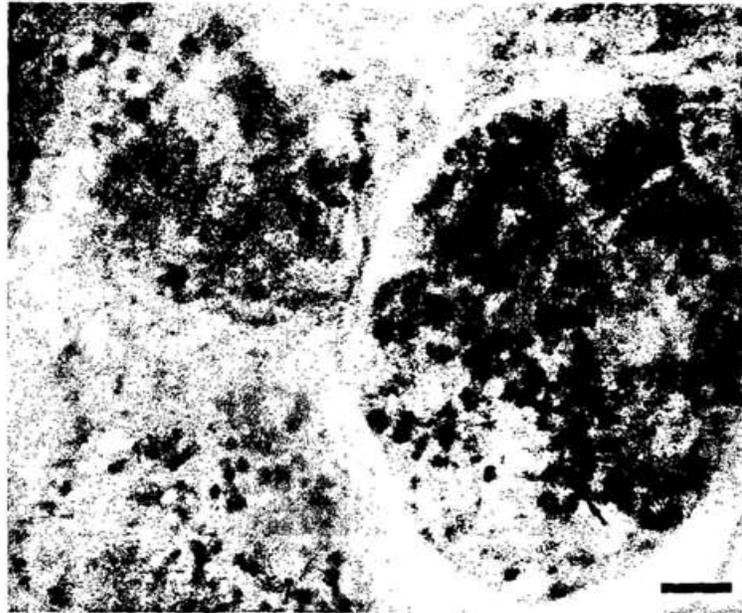
Fig. 15. Immunopositivity to anti-insulin antibody in each cluster

The tissue was fixed after onset of response to glucose and before complete degranulation. Dark spots (arrow) exhibit immunopositivity above control levels (compare with Fig. 5). The degree of staining reflects the number of β-granules remaining after stimulation. The staining was strong in a cluster to the right, but weak in two clusters to the left. Calibration, 5 μm.
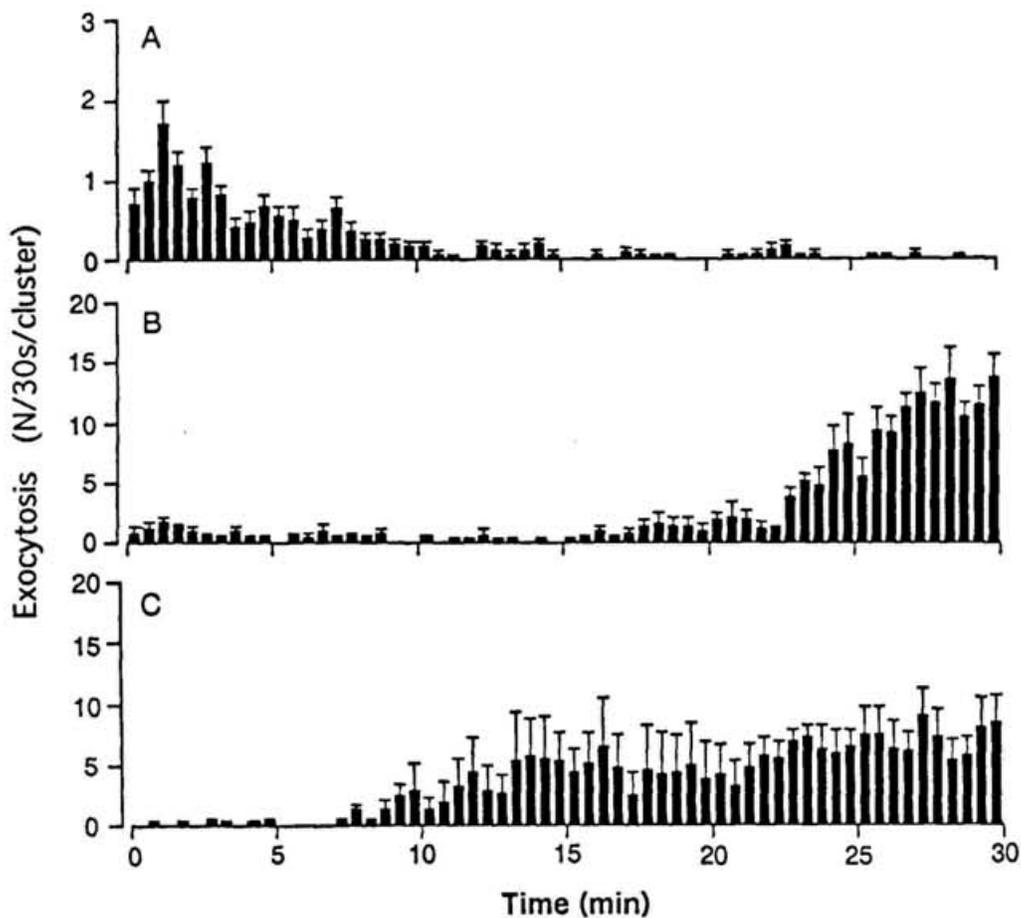
Fig. 16. Heterogeneity of response patterns in each cluster of β-cells

The patterns of glucose-induced exocytotic responses were classified to 3 types: an early phase response that occurred transiently in initial few minuites (A), an early phase response followed by a delayed response that occurred gradualy from 10 min after stimulation (B), and a delayed response not preceded by the early response (C). The number of quantal responses in every 30 s period in individual clusters was counted and averaged (expressed in frequency). The concentration of glucose in the medium was raised from 1.5 mM to 15 mM at the beginning of each histogram. Values are means ± S. E., n=30 for A, n=5 for B and C. Any responses found in a single cluster could be classified into one of these types.
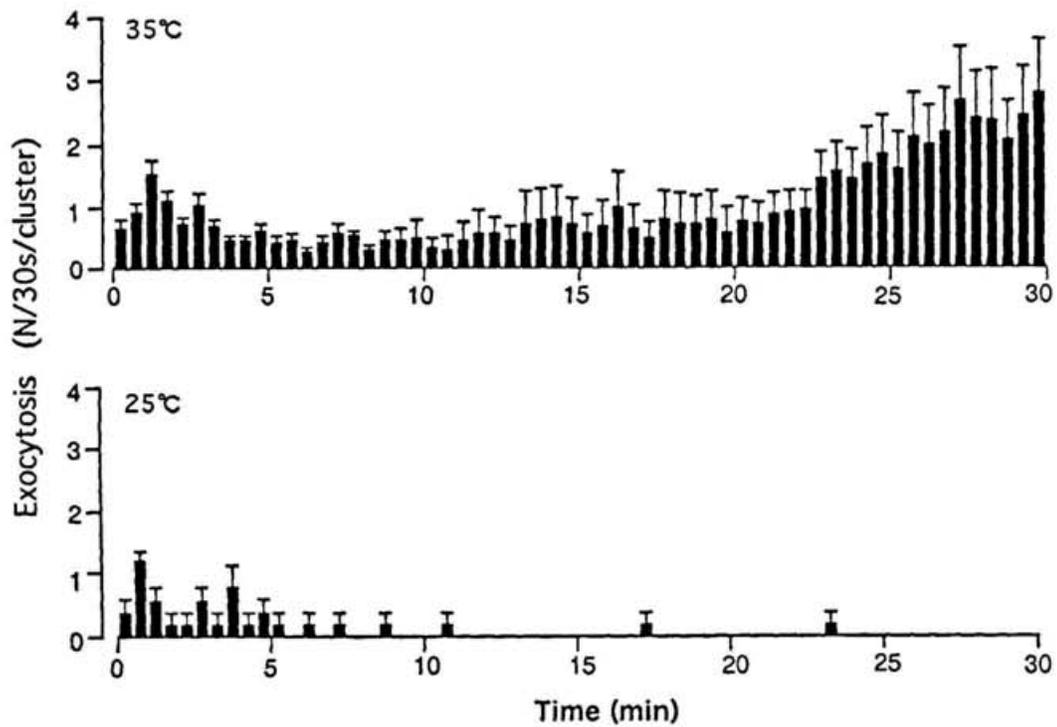
Fig. 17. Ensemble average of exocytotic responses observed in clusters of β-cells

Frequency histograms were obtained by summing all data from 40 clusters chosen randomly at 35 °C and from 5 clusters chosen from the peripheral region of the islet at 25 °C. The concentration of glucose in the medium was raised from 1.5 mM to 15 mM at the beginning of each histogram.
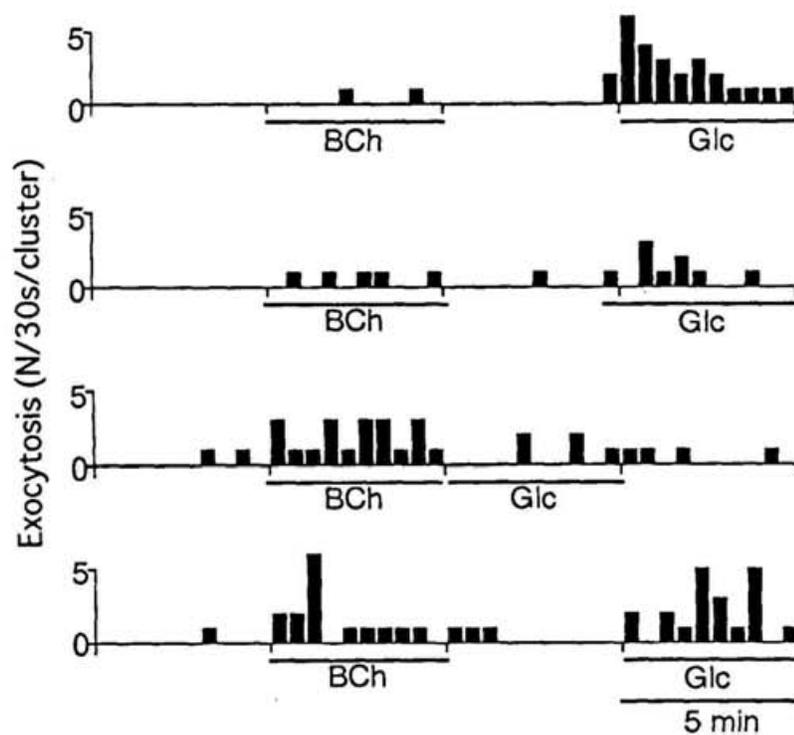
Fig. 18. Sensitivity to BCh in clusters of β-cells

Clusters were stimulated with 100 μM bethanechol (BCh) and 15 mM glucose (Glc). For identification of β-cells and for examining sensitivity to glucose, the concentration of glucose was raised to 15 mM before or after BCh application. Most clusters of β-cells showed some sensitivity to BCh, but at a variable extent. The number of quantal responses in every 30 s period in single cluster was counted and expressed as the height of bars.

Table 1. Early and late phase responses induced by glucose-stimulation in two classes of β-cell clusters

Population in cluster	Early phase (0-7 min)	Late phase (7-30 min)	Ratio (Late/Early)	n
Small	11.3 ± 1.1	19.9 ± 10.5	1.75	24
Large	9.7 ± 2.1	182.8 ± 45.7*	18.83	7
Non defined[†]	5.4 ± 1.7	41.2 ± 26.5	7.57	9
Total	9.8 ± 0.9	53.2 ± 14.8	5.4	40

Results are expressed in total number of exocytotic events (mean ± S.E.M.). Significance of differences was calculated by Student's t-test. *P<0.05. [†]The accurate number of cells could not be determined.

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DISCUSSION

I. *Insulin secretion from islet ß-cells*

Video-enhanced contrast differential interference contrast microscopy (Allen *et. al.*, 1981) provided a means to assess an ultrastructure of the living cell, and revealed physiological responses in the tissue maintaining its normal structure (Terakawa & Suzuki, 1991). By using this imaging technique, β-cells in an excised rat pancreas could be studied without any artificial staining. The distinction between β-cells and non-β-cells in an islet was difficult if based only on examination of their light microscopic appearance. However, the islet cells we observed showed several properties characteristic of β-cells: 1) The cells were sensitive to glucose, which is the most important physiological stimulus for the release of insulin from the β-cells (Ashcroft, 1980). 2) The degranulation occurred in the majority of cells in an islet, particularly in its central part, consistent with the fact that the β-cells form a homologous area in the center of the islet as a common feature of their configuration (Grube *et al.*, 1983). 3) The cells were immunopositive when examined using a peroxidase-conjugated anti-insulin antibody. Therefore, we securely conclude that the degranulating responses reflect secretory activity in β-cells. Some cells unresponsive to glucose stimulation in the peripheral region of the islet may be glucagon-containing α-cells.

In β-cells, it is not certain how much degranulation was due to exocytosis and how much was due to rupture of granules in the cytoplasm. The quantal responses of individual granules induced by glucose are indeed exocytotic, because series of the image are very similar to those found in chromaffin cells (Terakawa *et al.*, 1991), salivary cells (Segawa *et al.*, 1991), and the nasal secretory cells (Kamijo *et al.*, 1993). This idea is also supported

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by the finding: 1) The responses were induced by the elevation of intracellular Ca^{2+} concentration following the application of K^+-rich media or calcium ionophore-containing media, and also by application of sulfonylurea. These findings agree with the results of immunological assay of insulin release from the β-cells (Wollheim & Sharp, 1981; Henquin, 1992). 2) The granules remaining in these cells were clearly immunolabeled with an anti-insulin antibody, and the cytoplasm of these cells was not even after degranulation. Therefore, the quantal response of the granule as it appears in the video is an optical representation of the exocytosis of a β-granule. We consider that insulin was released anyway from β-cells to the extracellular space. This conclusion is further confirmed by the observation that hemolytic plaques due to the complement-mediated lysis were formed after glucose-induced degranulation (Fig. 6). Since the hemolytic plaque assay was incapable of assessing the time-course of insulin release from the pancreatic tissue, we could not firmly establish that the mode of secretion in β-cells is always exocytosis.

Motile events of granules in β-cells were studied earlier by cinemicrograph (Lacy et al., 1975; Somers et al., 1979). However, it was difficult to observe a rapid change in individual granules by that technique. In fact, signal amplification is important for direct visualization of such a response. The exocytotic events observed in individual β-granules were less obvious in real-time video display than those observed in chromaffin granules (Terakawa et al., 1991). Accordingly, some quantal events of single granules were barely discernible in still pictures (Fig. 4B). This is, perhaps, related to the small size of the dense core, and also to the slow rate of the release-process in β-granules. However, there is no ambiguity in counting the number of quanta by playing back the videotape owing to the high sensitivity of the human eye to a stepwise change in brightness (it is difficult, though, to count them directly through the ocular lenses of the microscope). The use of time-differ-

ential image processing makes the quantification even easier and more precise (Fig. 4C). In the critical step of exocytosis, granule movements relative to the plasma membrane are considered as a prerequisite for exocytosis and an integral part of stimulus-secretion coupling in islet β-cells as described previously (Lacy *et al.*, 1968). However, in our study, there was no indication of such granule movement before exocytosis. The continuous movement of β-granules at an extremely low rate suggests that some granules which are already docked to the plasma membrane at the time of stimulation undergo exocytosis. The mechanism that initiates exocytosis directly may be a molecular reaction taking place at the docking site only.

A long time after the first observation of living islet cells *in situ* (McCuskey & Chapman, 1969), direct morphometric quantitation of insulin secretion became possible. Our quantitative analyses indicate that the quantal events in each cell induced by a brief challenge of glucose were rather few in number. This leaves ample room for a number of ways to increase the frequency of such events. Repetitive challenges with glucose or with potentiator of secretion, for example, clearly showed an augmentative effect (Fig. 7, B and C). Simultaneous signalings of glucose with other stimulants such as acetylcholine (Ahren *et al.*, 1986), arginine (Schmidt *et al.*, 1992), glucagon (Pipeleers, 1984), and pituitary adenylate cyclase activating polypeptide (Yada *et al.*, 1994) make it highly possible to enhance the response *in vivo*. Continuous depolarization of β-cells *in vivo* may result in a large response. It is known that long continuous stimulation of a pancreatic tissue with glucose induces much larger biphasic secretion of insulin than a short stimulation (Curry *et al.*, 1968). Taking advantage of the high sensitivity, high stability, and high temporal resolution of video-microscopy, we are currently studying the enhancing effect of various modes of glucose stimulation and the intracellular signaling pathway underlying the enhanced insulin secretion.

II. *Morphology of β-cell clusters*

The ultrastructure of pancreatic islets was studied by electron microscopy (Goldstein & Davis, 1968; Bonner-weir, 1988). However, the spatial organization of β-cells in an islet has not been studied in the living tissue while it is maintaining physiological functions. The VEC-DIC microscopy made it possible to assess both the fine structure and the secretory response in various functional tissues (Terakawa, 1989). It was one of the great advantages in the DIC-microscopy that cross sectional images of the islet were obtainable only by changing the focus without any invasive procedure, for example, fixation, slicing and staining. We observed images of endocrine β-cells in an excised pancreatic tissue and in an intravitally circulated pancreas. These images revealed that the islet is filled with clusters of 8 - 16 cells with small clusters located in a core region and large one in a mantle region (Figs. 9, 10, and 12).

It has been conceived that rows or columns of pancreatic endocrine cells are arranged and oriented around a central capillary, and form a tube-like or cord-like structure (Bonner-Weir, 1988). The relationships between the cluster and the microvasculature in the islets were most apparent by intravital imaging of a normally circulated pancreas where continuously moving erythrocytes appeared as distinctively dark objects marking capillaries so obviously. Our observations clearly indicate that the blood capillaries form some frames for the cluster rather than an axis for it. The size of the cluster is comparable to the spaces between the glomerular net of an islet (Bonner-Weir, 1982). Where there was no capillary, the border between clusters was barely discernible by the video-enhanced contrast microscopy (Figs. 9 and 11). Fluorescent staining (Fig. 10) better highlighted the border between them and also the interstitial spaces. The reason why the islet cells have been considered to date as continuous masses (Jörns *et al.,* 1988) may be that a three dimensional scanning capability was not available with a resolution high enough to visualize the border between clusters.

In our experiment, a considerable number of islet cells could be extensively degranulated by prolonged glucose stimulation (i.e. 15 mM glucose, < 60 min), which provided us an opportunity to elucidate the structure of the clusters in detail. After massive degranulation, the spaces between cells and clusters were widened, and the adverse effect of light scattering by granules on the DIC optics was decreased. The clusters were not clear in the islet isolated by the collagenase digestion method. One reason could be a lack of massive degranulation in such an islet and another could be a loss of clustering itself due to depletion of collagen fibers. It was suggested that the tight junctions possibly sealing areas of intercellular spaces are uncommon and small, if any, in rat Langerhans islets *in situ* (In't Veld *et al.,* 1984). Moreover, an intercellular canalicule system was described to separate β-cells rather widely (Fujita *et al.,* 1981). Therefore the clustered structure could be maintained by collagen fibers and by some adhesion molecules other than tight junctions.

We found that the number of cells in one cluster tended to be binarized —i.e. a small-sized cluster contained about eight cells and a large-sized cluster contained about sixteen cells. Further, there was a tendency that the locality and physiological responses of the clusters in the islet depended on the cluster size (see DISCUSSION IV). This may be pertinent to the early finding that β-cells in the central region and peripheral region of an islet face different cellular and hormonal environments (Orci & Unger, 1975). The clustered arrangement of islet cells is not a novel or unexpected concept (Jörns *et al.,* 1988). Very similar clusters were also noted in dissociaton culture of β-cells (Taylor *et al.,* 1994*)*. The present study provides firm evidence for clustering of β-cells *in situ* and in the three-dimensional space within the islet, and a concept of the particular intra-islet organization or compartmentalization has been solidly established (see Fig. 19).

III. *Functional heterogeneity of the ß-cell cluster*

In addition to the structure of clustering, we observed also that the secretory activity of clusters of β-cells has their individuality. Diversity in the function of β-cells has been studied by various techniques used at various levels. In regard to the secretory activity, the heterogeneity was examined by measuring the amount of substances released biochemically (Salomon & Meda, 1986), immunologically (Van Schravendjk *et al.,* 1992) in isolated islet cells, and morphologically (Stefan *et al.,* 1987) in fixed preparations. The present technique allowed us to examine the variation of secretory activities in an excised tissue preparation which is morphologically intact and free from the factors that may modulate the effect of glucose.

The patterns of exocytotic responses induced by glucose were quite similar when compared among cells belonging to the same cluster, but were quite variable when compared among clusters. This defines the functional personality of the cluster. Personalities of clusters of β-cells were noted previously in glucose-induced changes in intracellular Ca^{2+} concentration in the mouse (Taylor *et al.,* 1994). To confirm the heterogeneity of clusters, simultaneous imaging in a whole islet may be ideal. However, the secretory granules cannot be resolved in a wide-view observation at a low magnification. At present, we can only compare the heterogeneity between neighbouring clusters in many preparations. The partial pressure of oxygen (pO_2) affects the rate of insulin secretion, especially that of second-phase response (Dionne *et al.,* 1993). In our observation, the second-phase response appeared only in 20% of the clusters usually located in the periphery of the islet. It might be possible that low pO_2 in the central region of islet suppressed the second-phase response in clusters there. However, the heterogeneity of β-cell cluster is due not only to a difference in oxygen supply, since the variations were observed in the clusters neighboring to each other and the late-phase responses were

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occasionaly observed in clusters located in the center of the pancreatic tissue preparation. Many clusters located at the cut edge of the tissue also showed no second-phase response.

The heterogeneity of clusters was best elucidated by glucose stimulation. The heterogeneity was suppressed when the clusters were stimulated with A23187-containing solution or K^+-rich solution both of which elevate the concentration of intracellular Ca^{2+}. Early work on the electrophysiological properties of islet cells showed that the threshold for glucose-induced electrical activity is variable from cell to cell (Dean & Matthews, 1970). It is suggested that the differences in responses of individual β-cells are closely related to the cellular handling of glucose (Kiekens et al., 1989). Therefore, the heterogeneity of clusters could be due to different metabolic responses to glucose and/or to glucose-induced electrical activities of their member cells. Further studies are required to determine whether β-cells bearing similar properties in a cluster are originated from a single stem cell.

Our results do not exclude the presence of extrinsic factors that may affect the clusters differently. The cholinergic nerve terminals have been identified in the islets, and current evidence suggests that the main function of the cholinergic innervation is to enhance insulin secretion after food ingestion (Ahren et al., 1986). Our results indicate that clusters have a various sensitivity to the parasympathetic neurotransmitter. Therefore, the heterogeneity of β-cells may partly arise from different neuronal environments and also from their different responsiveness to neurotransmitters. The clusters are likely to be under individual neuronal control for modulation of the secretory response.

IV. *Physiological significance of the ß-cell cluster*

A mass measurement indicated that a considerable pool of insulin-containing granules was always reserved in the ß-cells and less than 10 - 20 percent of the insulin stored was released upon maximal stimulation with glucose (Halban, 1982). In our observation at a cellular level, a small number of clusters, less than 20 per cent, showed the late phase response with a full degranulation in 60 min. On average, the value is consistent to the early estimation, but the significance is totally different. It was reported that the rate of insulin biosynthesis in the ß-cell is rapid and appears to be largely regulated by the extracellular glucose concentration (Rhodes & Halban, 1987). We could not observe new synthesis of secretory granules in the fully degranulated clusters in 60 min. It is not clear, at the moment, whether this lack of synthesis in some clusters of ß-cells is due to a lack of essential factors, such as nutrients, hormones, and neurotransmitters in our experimental medium or to the natural fate of some cells to turnover.

A study on dissociated cells suggested that the organization of ß-cells in culture, single or aggregated, may affect the amplitude of their secretory response (Pipeleers *et al.*, 1992). In *vitro* studies on the kinetics of glucose-induced insulin release showed a rapid initial phase and a slowly ascending late phase (Curry *et al.*, 1968). The present study links these observations by presenting evidence that the biphasic insulin secretion is a result of the heterogeneous activity of ß-cell clusters (Fig. 16 and 17). Clusters in an islet and aggregations in culture may share a common advantage for an efficient and endurable secretion. It is widely believed that ß-cells contain two pools of secretory granule; the readily releasable pool that is for the early transient phase, and the newly mobilized cytoplasmic pool that is for the late sustained phase (Gold & Grodsky, 1984). Contrary to this idea, the two components are derived from different clusters, and the delayed response

is not due to mobilization of granules from one pool to the other in the same cluster. Two classes of clusters could be a result of differentiation between two necessary functions: one for a rapid onset of release and another for long-lasting release. These functions are developed in relation to the number of member cells and the location in the islet. The clusters of β-cells near the center of an islet is mostly responsible for the rapid insulin secretion during brief stimulation, whereas those in the periphery of the islet significantly contribute to the large and delayed insulin release after prolonged stimulation. Thus, the individual islet β-cells heterogeneously contribute to a tight and long-lasting control of glucose homeostasis.
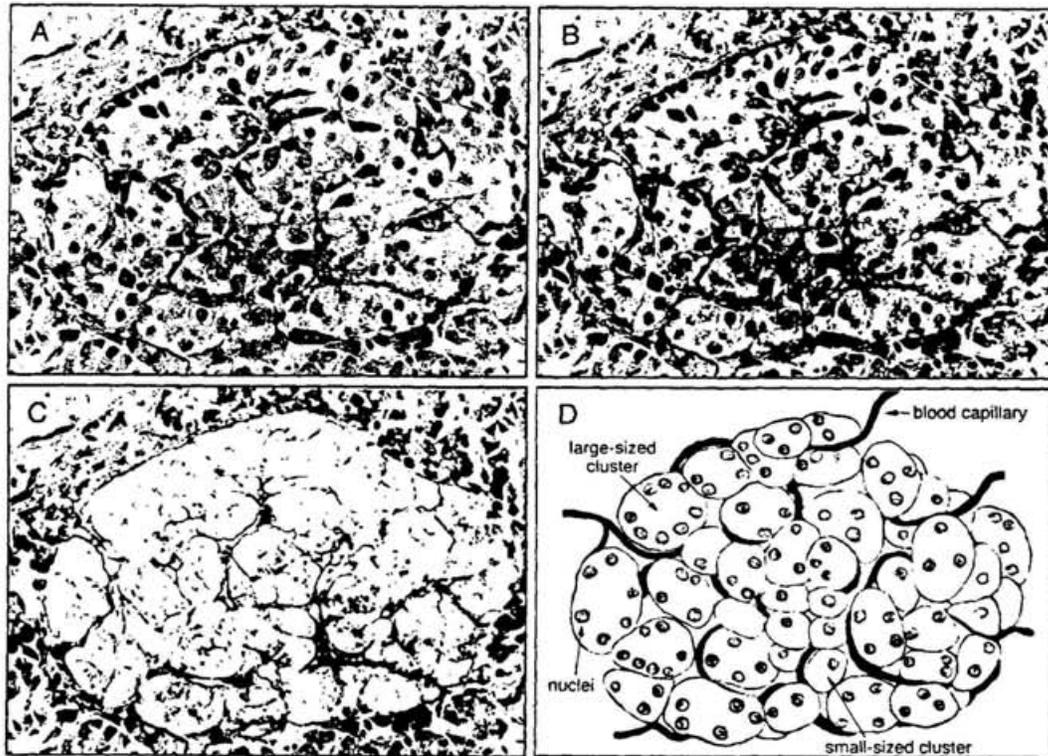
Fig. 19. Clustered model of islet cells

A model of intra-islet compartmentalization in Langerhans was illustrated by modifying a conventional light microscopic image (Fujita, 1964). **A**, Fujita's original image of an islet stained by argentation and digitized at an 8 bit resolution. In addition to argyrophilic cells, some frame-like structures are recognizable in the islet. **B**, binary image of A (threshold value = 140). Many of filled circles (i.e., arrows) indicate nuclei. **C**, image obtained by deleting argyrophilic cells and all of the nuclei from B. Some compartmental structure remained. **D**, conceptual illustration of the Langerhans islet drawn on the basis of the image in C. The borders between clusters were partly outlined from the compartments in C and partly from the location of nuclei, that was usually in the periphery of a cluster. No border between islet cells in a cluster is visible *in situ* by video microscopy. Capillaries were drawn in large spaces between clusters. Two classes of the cluster can be distinguished by their sizes. Small-sized clusters are dense in the core of the islet. Large-sized ones tend to form a mantle of the islet.

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