Ethidium Bromide-induced Inhibition of Mitochondrial Gene Transcription Suppresses Glucose-stimulated Insulin Release in the Mouse Pancreatic β-Cell Line βHC9*

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Takaki Hayakawa, a,b,c Mitsuhiro Noda, a,c,d,e Kazuki Yasuda, a,d,g Hiroshi Yorifuji, h
Shigeki Taniguchi, i Ichitomo Miwa, i Hiroshi Sakura, i Yasuo Terauchi, a Jun-ichi Hayashi, j
Geoffrey W. G. Sharp, a Yasunori Kanazawa, a Yosuo Akanuma, a Yoshio Yazaki, a
and Takashi Kadowaki a

From the “Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan, a,b,c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, u, v, w, x, y, z, a, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, u, v, w, x, y, z

Recently, a mitochondrial mutation was found to be associated with maternally inherited diabetes mellitus (Kadowaki, T., Kadowaki, H., Mori, Y., Tobe, K., Sakuta, R., Suzuki, Y., Tanabe, Y., Sakura, H., Awata, T., Goto, Y., Hayakawa, T., Matsuoka, K., Kamawori, R., Kamada, T., Horai, S., Nonaka, I., Hagura, R., Akanuma, Y., and Yazaki, Y. (1994) N. Engl. J. Med. 330, 962–968). In order to elucidate its etiology, we have investigated the involvement of mitochondrial function in insulin secretion. Culture of the pancreatic β-cell line, βHC9, with low dose ethidium bromide (EB) (0.4 μg/ml) for 2–6 days resulted in a substantial decrease in the transcription level of mitochondrial DNA (to 10–20% of the control cells) without changing its copy number, whereas the transcription of nuclear genes was grossly unaffected. Electron microscopic analysis revealed that treatment by EB caused morphological changes only in mitochondria and not in other organelles such as nuclei, endoplasmic reticula, Golgi bodies, or secretory granules. When the cells were treated with EB for 6 days, glucose (20 mM) could no longer stimulate insulin secretion, while glibenclamide (1 μM) still did. When EB was removed after 3- or 6-day treatment, mitochondrial gene transcription recovered within 2 days, and the profiles of insulin secretion returned to normal within 7 days. Studies with fura-2 indicated that in EB-treated cells, glucose (20 mM) failed to increase intracellular Ca2+, while the effect of glibenclamide (1 μM) was maintained. Our system provides a unique way to investigate the relationship between mitochondrial function and insulin secretion.

The mitochondrial genome of mammalian cells encodes 13 polypeptides, two ribosomal RNAs, and 22 transfer RNAs (for a review, see Ref. 1). The mitochondrion is believed to be an organelle derived from a genetic component(s) of microorganisms, and thus its replication, transcription, and translation system has been developed on its own basis, although several nuclear genome-encoded proteins are also essential for these systems. Mitochondrial proteins involved in oxidative phosphorylation are composed of enzyme complexes (I–IV) and cooperate with a number of nuclear genome-encoded proteins for ATP production.

Currently, the following hypothesis is widely accepted as a major part of the glucose-signaling pathways for insulin secretion in pancreatic β-cells. First, glucose is transported into the pancreatic β-cells and metabolized through the glycolytic pathway and Krebs cycle. ATP is produced by oxidative phosphorylation within the mitochondria. The increased ATP and decreased ADP concentrations cause depolarization of the plasma membrane via closure of ATP-sensitive K+ channels. Depolarization activates the voltage-dependent calcium channels and increases [Ca2+]. This increase in [Ca2+] stimulates exocytosis of insulin granules from the pancreatic β-cells (for a review, see Ref. 2). Several lines of evidence suggest an important role for the mitochondria in this pathway. First, ATP-sensitive K+ channels are believed to “sense” ATP produced by mitochondria and to convert the information into depolarization of membrane potential. The presence of this type of channels in pancreatic β-cells was first demonstrated by electrophysiological approaches (3, 4) and recently established on a molecular basis (5, 6). Second, 2-ketoisocaproate, which is metabolized intramitochondrially, exerts the same stimulatory effects on insulin secretion as glucose does (7, 8). Third, it has been shown that mutations of mitochondrial DNA are associated with diabetes mellitus (9–11). We have recently pointed out that diabetic patients with an A to G mutation at position 3243 in mitochondrial DNA (base pair 3243 mutation) have reduced insulin secretory capacity rather than peripheral insulin resistance (12). In addition, Hess et al. (13) and Chomyn et al. (14) showed by in vitro study that this mutation results in mitochondrial transcriptional and translational defects. However, there are few reports that have demonstrated a correlation between mitochondrial (dyse)function and insulin secretion directly.

To examine the relationship between mitochondrial function and the stimulation of insulin release, we employed a newly developed insulin-secreting cell line, βHC9 (15–17), and cul-
tured the cells with ethidium bromide (EB), an inhibitor of the synthesis of DNA and RNA. To date, few detailed studies on the role(s) of mitochondrial function in glucose signaling have been performed. In one of these, in which bis-4-piperidylidene-dichloride was employed to establish a ρ0 cell line in the MIN6 insulin-secreting cell, it was shown that the presence of mitochondria was essential for glucose-stimulated insulin release (18), as discussed below.

It has been reported that EB, a reagent that inhibits DNA/RNA synthesis, affects transcription/replication of extrachromosomal genetic materials more specifically than those of chromosomal genes (19–21). Recently, human cell lines lacking mitochondrial DNA (ρ0 cells) were established by long-term treatment with low concentrations of EB (22, 23). Hayashi et al. (24) also showed that a specific inhibition of replication, transcription, and translation of genes takes place with mitochondrial DNA when a mouse fibroblast cell line was treated with EB for up to 7 days.

In this report, we investigated the effects of EB treatment and the virtual elimination of mitochondrial transcription on glucose-stimulated insulin secretion and on [Ca2+], EB treatment blocked the effect of glucose to increase [Ca2+], and to stimulate insulin secretion. The effect of EB was completely reversed after removal of the EB. These results provide strong evidence that mitochondrial function is crucial for the stimulation of insulin release by glucose.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes and DNA-modifying enzymes were purchased from Takara Shuzo Co. (Kyoto, Japan), EB, bovine serum albumin (BSA), nifedipine, and fura-2 were obtained from Sigma, and [α-32P]dCTP was from Amersham Pharmacia Biotech. Glibenclamide was provided by Hchoct Lab., Co., Ltd.

**Cell Culture**—βHC9 was a kind gift from Dr. D. Hanahan (University of California). βHC9 cells were maintained in Dulbecco’s modified Eagle’s medium containing 25 mM glucose, 0.11 mg/ml pyruvate, and 0.05 mg/ml serum albumin; GK, glucokinase; HK, hexokinase.

**Glucose-phosphorylating activities by hexokinase (HK) and glucokinase (GK) of either control or EB-treated cells on day 4 were determined fluorometrically as described previously (29, 30).**

**Electron Microscopic Analysis**—The control and EB-treated cells were fixed overnight with 2.5% glutaraldehyde in 0.1 m sodium phosphate buffer (pH 7.5). They were postfixed with 2% Oso4 in the same buffer followed by en block staining with 1% uranyl acetate. After dehydration with a graded series of ethanol, they were substituted by propylene oxide and embedded in Spurr’s low viscosity resin. Silver to gold sections were cut and examined with a JEOL 1010 electron microscope (JEOL, Tokyo, Japan).

**Insulin Secretion and Content**—The control and EB-treated cells were suspended in 12-well plates. Each well was washed twice with 1 ml of phosphate-buffered saline and then incubated with 1 ml of Hanks’-BSA medium composed of Hanks’ buffered saline containing 0.2% BSA and 10 mM HEPES, pH 7.5, plus 0.1 mM glucose in 5% CO2 at 37 °C. After incubation for 2 h, the medium was replaced with 1 ml of the Hanks’-BSA medium containing various secretagogues. For the experiment shown in Fig. 6, Krebs-Ringer bicarbonate buffer was used instead of Hanks’ buffer. After incubation for 2 h, the medium was collected and centrifuged at 6000 rpm for 2 min, and the supernatant was collected and stored at −20 °C until radioimmunoassay for insulin concentration. For determination of insulin content in the cells, each well was washed twice with 1 ml of phosphate-buffered saline and suspended in 1 ml of a solution containing 74% ethanol and 1.4% HCl and kept at −20 °C for 18–24 h. Then the supernatant was collected and stored at −20 °C until assayed. Radioimmunoassay kits (Shionoria; Shionogi & Co., Ltd., Japan) were used for the determination of insulin levels using mouse insulin as standard. Total cellular proteins were extracted by boiling the trypsinized cells in 1% SDS, and protein concentration was determined by the method described by Lowry (31) with BSA as a standard.

**Measurement of [Ca2+]i**—Control and EB-treated cells were cultured on a glass coverslip in a 6-cm diameter dish. Each dish was washed twice with 2 ml of phosphate-buffered saline and incubated with 4 ml of Hanks’-BSA medium for 40 min. The cells were then loaded with fura-2 for 40 min in 4 ml of the Hanks’-BSA medium containing 4 μM fura-2 acetoxymethylester (added from a 1 mM stock solution in dimethyl sulfoxide), 0.05% CO2 at 37 °C. After incubation for 40 min, the cells were loaded with fura-2 and then prepared for measurements. Epi-illumination at 340- or 360-nm wavelength was converted into a ratio signal fluorescence by 340-nm wavelength was measured before and then 15 and 30 min after stimulation by replacement with the Hanks’-BSA medium containing 20 mM glucose or 1 μM glibenclamide. All measurements of [Ca2+]i were carried out at 37 °C. A dual excitation digital imaging system (Argus 100 image analysis system; Hamamatsu Photonics Inc., Japan) was used for the measurement. Epi-illumination at 340- or 360-nm wavelength from a mercury lamp was used to excite fura-2. Emitted fluorescence was magnified through a fluorescence objective lens (X10; Nikon Inc., Japan) taken by a silicon-intensified tube camera, and then converted into a digitized value of 256 gray levels at each pixel. Paired fluorescence images by 360-nm wavelength excitation were taken before and after a single measurement and interpolated each time. The signal fluorescence by 340-nm wavelength was converted into a ratio divided by the value of interpolated 360 nm at each pixel to yield a pseudocolor image of [Ca2+]i. Conversion from the ratio to [Ca2+]i was performed using a Ca2+-EGTA standard (Molecular Probes, Inc.). Data were expressed as two-dimensional mean values of [Ca2+]i, which were calculated by averaging the values of all of the pixels over the cellular area.

**Statistical Analysis**—Data shown are means ± S.D. of triplicate observations in a single representative experiment unless otherwise indicated.

**RESULTS**

**Characteristics of βHC9 Cells upon Insulin Secretion**—Table 1 summarizes the characteristics of insulin secretion and insulin content in βHC9 cells. Analyses were carried out when cells reached 70–90% confluency. Twenty mM glucose induced a substantial increase in insulin secretion from the basal level (0.1 mM glucose alone). Half-maximal effect of glucose on insulin secretion was obtained at 1–1.5 mM (shown in Table 1), consistent with the results of Radvanyi et al. (15) and Noda et al. (17). Glibenclamide (1 μM), KCl (20 mM), and 2-ketoisocaproate (10 mM) also stimulated insulin secretion. In other experiments, it was shown that nifedipine (100 nM), an inhibitor of L-type voltage-dependent calcium channels, inhibited the stimulation of insulin secretion by glucose (20 mM) or glibenclamide (1 μM).
cate observations in a single representative experiment are shown here. Means ± S.D. of triplicate observations in a single representative experiment are shown here. Means ± S.D. of triplicate observations in a single representative experiment are shown here. Means ± S.D. of triplicate observations in a single representative experiment are shown here.

Effect of EB on Mitochondrial Replication and Transcription—βHC9 cells were incubated for 4 days with various concentrations of EB in Dulbecco’s modified Eagle’s medium supplemented with pyruvate and uridine, which was previously shown to be essential for the growth of ρ0 cells (cells lacking mitochondrial DNA) (22). Fig. 1A shows changes in gene transcription in EB-treated cells measured by Northern blot analyses. The mitochondrial DNA probe used for these analyses detected several transcripts (two rRNAs, 10 tRNAs, and four peptides), including two major hybridized bands. The relative quantity of the mitochondrial transcripts was calculated in comparison with the radioactivity of 16 S transcript(s). These analyses showed that the reduction of mitochondrial transcription by EB treatment occurred in a concentration-dependent manner, and the mitochondrial transcription level of EB-treated (0.4 μg/ml) cells was reduced to 10–20% of the control cells, whereas the transcription of the insulin gene was not affected. Transcription of β-actin was also unaffected as described below.

The copy number of mitochondrial DNA was not changed by EB treatment at the concentrations below 2.5 μg/ml (Fig. 1B); the cells could not grow at EB concentrations over 6 μg/ml.

Time Course Analysis of Transcription of Nuclear and Mitochondrial Genes in EB-treated Cells—We examined the temporal profiles of mitochondrial transcription and insulin secretion 1–6 days after the addition of EB. On days 4–6, the growth of EB-treated cells was significantly retarded (see below). On days 3–6, mitochondrial transcription levels of the control cells were enhanced, whereas those of EB-treated cells were inhibited by 80–90% compared with control cells (Fig. 2). In contrast, transcription of the insulin gene showed little change between treated and untreated cells (Fig. 2). Expression of “housekeeping” genes also seemed to be unaffected by EB treatment when the messenger RNA of β-actin was assessed on day 0, 2, 4, and 6 by reverse transcription-polymerase chain reaction using the Mouse β-Actin Control Amplimer Set (CLONTECH, Palo Alto, CA) (data not shown). Analysis by reverse transcription-polymerase chain reaction of cells treated with EB for 6 days also showed that transcription of major glucose-sensing enzymes, GK and HK-I, was not altered significantly (data not shown). In accord with this, glucose-phosphorylating activities by HK and GK were not changed between EB-treated and control cells on day 4 (Fig. 3).

Electron Microscopic Analysis—On electron microscopic analysis (Fig. 4), βHC9 cells contained electron-dense secretory granules in their cytoplasm, which probably represented insulin-containing granules, because fluorescence microscopy of anti-insulin antibody to these cells revealed fine granular staining in the cytoplasm (data not shown). Although dead or damaged cells were occasionally seen after EB treatment, most of the cells looked morphologically intact, as also shown by phase-contrast microscopy (data not shown). The only prominent finding observed in these cells was the frequent appearance of ring- or cup-shaped mitochondria (Fig. 4A). No changes were found in other organelles including Golgi apparatus or dense core granules.

Time Course Analysis of Insulin Secretion in EB-treated Cells—Basal insulin secretion (0.1 mM glucose) of the control cells maintained a constant low level during cell proliferation. The stimulatory effect of glucose (20 mM) on insulin secretion was gradually increased, and the maximal effect was observed on day 6 (confluency had reached 70–90% at this time).

| Secretagogue | Insulin secretion | Insulin content |
|--------------|-------------------|-----------------|
|              | ng/mg cellular protein/2 h | ng/mg cellular protein |
| 0.1 mM glucose | 50 ± 13 | 3390 ± 879 |
| With 20 mM glucose | 810 ± 121 | ND* |
| With 1 μM glibenclamide | 1520 ± 232 | ND |
| With 10 mM ketoneoctoate | 100 ± 5 | ND |
| With 20 mM KCI | 490 ± 161 | ND |

* ND, not done.
similar temporal profile to that of glucose-induced insulin secretion was observed with glibenclamide (1 \( \mu \)M)-stimulated insulin release (Fig. 5A). In contrast, in EB-treated cells, glucose-stimulated insulin release was completely abolished by day 5–6, although the stimulatory effect of glibenclamide was still observed (Fig. 5B). The insulin content of EB-treated cells was higher than those of the control cells (tested on days 4 and 6; Table II). In addition, glucose-phosphorylating activity by either HK or GK was unchanged between EB-treated and control cells (on day 4; Fig. 3). Reverse transcription-polymerase chain reaction analysis of these two enzymes of the cells treated with EB for 6 days showed similar expression levels to those of control cells (data not shown).

To examine the effects of EB treatment on glucose responsiveness in more detail, we evaluated the concentration-response characteristics of glucose-stimulated insulin secretion after 6 days of EB treatment. As shown in Fig. 6 and Table II, increase in basal secretion and decrease in stimulated release both contributed to the loss of fold increase by the treatment. Fig. 6 also shows that half-maximal effect of glucose on insulin secretion was achieved at 10–15 mM.
Although glucose responsiveness and insulin content of the βHC9 cell line underwent some changes over different passages (see Ref. 18, Table II), and Fig. 6), the loss or decrease of glucose-stimulated insulin release due to EB treatment was absolutely reproducible in more than 50 experiments ranging over more than 15 passages, while there was no decrease in the responsiveness to glibenclamide. The effect of EB on insulin content was also observed by the same treatment repeatedly.

Reversible Change of Mitochondrial Transcription and Properties of Insulin Secretion—After 3 days of treatment with EB, cells were returned to EB-free culture medium, and mitochondrial transcription and insulin secretion were examined. As described earlier, mitochondrial transcription of cells treated with EB for 3 days was only 10–20% of that of the control cells. After removal of EB from the medium, mitochondrial transcription was slightly raised after 1 day and restored at 2 days (Fig. 7). The transcription levels of the insulin and β-actin genes were increased 2–3-fold on days 2–5. Importantly, the effects on insulin secretion by glucose started to recover 3 days after the removal of EB and were completely reversed in 5–7 days (day 6) (Fig. 8). This 3-day delay in recovery could be attributable to the time course of restoration of mitochondrial transcription (see “Discussion”). Mitochondrial transcription and insulin secretion by glucose were also restored when EB was removed after 6 days of EB treatment (data for transcription not shown). This reversible change in insulin secretion was also observed with a later passage (passage 29) as shown in Table III.

Changes in [Ca2+].—Changes in the [Ca2+]i in response to 20 mM glucose or 1 μM glibenclamide were examined in control and EB-treated cells using fura-2 as the indicator (Fig. 9). As shown in Fig. 9, the growth of EB-treated cells was significantly retarded compared with the control cells on day 6.

In control cells that were cultured for 7 days and reached 70–90% confluency, [Ca2+]i (the mean value for the cellular area) increased up to ~150 nM after a 15-min stimulation by 20 mM glucose. A similar increase was observed with the stimulation by 1 μM glibenclamide for 15 min. In cells cultured with EB for 6 days, the basal [Ca2+]i was significantly lower than that of the control cells (61 ± 10 versus 90 ± 16 nM, p < 0.05 by Student’s t test; both values from five independent observations); the increase induced by 20 mM glucose in the control cells was not seen in the EB-treated cells, whereas that due to 1 μM glibenclamide was similar to that of the control cells. These results were reproducible in three independent experiments. Similar results were obtained during 30-min stimulation by 20 mM glucose or 1 μM glibenclamide. Restoration of the [Ca2+]i response to glucose by removal of EB after a 3-day treatment was also verified.

**FIG. 6.** Insulin secretory response to glucose of the EB-treated and control βHC9 cells. Secretion from βHC9 cells by various concentrations of glucose was evaluated after 6-day treatment by EB (0.4 μg/ml) as described under “Experimental Procedures.” Means ± S.D. of triplicate observations in a single representative experiment are shown here. Solid line, control cells; broken line, EB-treated cells.

**FIG. 7.** Reversible mitochondrial transcriptional change by EB treatment. βHC9 cells were plated in a 10-cm dish at a density of 3 × 10⁴/cm² and cultured overnight, and then 0.4 μg/ml EB was added. After cultivation for 3 or 6 days, the medium was replaced by EB-free medium, and the total RNA was prepared at the indicated time. Northern blot hybridization (20 μg of the total RNA) was performed as described in the legend for Fig. 1. Mouse mitochondrial DNA was used first and then the membrane was rehybridized with rat insulin I cDNA plus human β-actin cDNA. 12 S products are shown for the transcription of mitochondrial DNA. The left lane shows the total RNA from control cells cultured for 3 days.

**FIG. 8.** Reversible change of insulin secretory characteristics. βHC9 cells were plated in a 12-well dish at a density of 3 × 10⁴/cm² and cultured overnight, and then 0.4 μg/ml EB was added. After cultivation for 3 or 6 days, the medium was replaced by EB-free medium and subsequently cultured for the indicated time. Control cells (day 0) were cultured for 3 days without EB. Insulin secretion stimulated by 20 mM glucose or 1 μM glibenclamide for 2 h was measured. Data presented are mean ± S.D. (n = 3).
Reversibility of the changes caused by EB after its removal at passage 29

| Treatment                  | Basal (0.1 mM glucose) | With 20 mM glucose | With 1 μM glibenclamide |
|----------------------------|------------------------|--------------------|-------------------------|
| 6-day EB                   | 2.59 ± 0.67            | 3.26 ± 0.90        | 6.86 ± 1.15             |
| 6 days after removal of 6-day EB | 1.15 ± 0.22            | 4.46 ± 0.29        | 6.06 ± 0.43             |

- Difference not significant between this test group and 0.1 mM glucose.
- *p* < 0.01 for the difference between this test group and 0.1 mM glucose.

show that synthesis of insulin is even enhanced in the EB-treated cells compared with the control cells. One possible explanation for this phenomenon is an enhanced posttranslational mechanism(s), since long term exposure to high glucose has been reported to elevate insulin biosynthesis, which outgrows the increase in mRNA content of insulin (36). In EB-treated cells, possible elevated glucose 6-phosphate levels, as a result of unchanged GK and HK activities and suppressed mitochondrial function, might well affect this level of regulation of insulin biosynthesis, since similar phenomena were reported in other cell types (37, 38). The inhibitory effect of EB on mitochondrial transcription was preceded by its effect on mitochondrial replication (Fig. 2), suggesting that the remaining transcription level (10–20%) was enough to maintain mitochondrial replication.

When EB was removed, all of the changes induced by the treatment (cell growth, insulin secretory profiles, and Ca\(^{2+}\) dynamics) were reversed, along with the restoration of mitochondrial transcription. This suggests that the changes observed during this treatment were due to mitochondrial dysfunction and that the treatment caused no irreversible effects on chromosomal genes. In fact, recovery of the insulin secretory profile was in good accord with the restoration of mitochondrial transcription. As shown in Fig. 7, it needed 2 days for the mitochondrial transcription to recover fully, and the secretory response to glucose started to recover 1 day later (Fig. 8). This 1-day delay might well be due to the time required for protein synthesis following the recovery of mitochondrial transcription.

During treatment with 0.4 μg/ml EB for 6 days, the growth of βHC9 cells was retarded. This contrasts with the previous report by King and Attardi (22) that β\(^{0}\) cells lacking mitochondrial DNA, derived from the human osteosarcoma cell line 143B, did not show growth inhibition compared with the parent cells when pyruvate and uridine were supplemented in the medium. Although the reason for this difference is unclear, one possible explanation could be that βHC9 cells (and possibly normal β-cells) are more “fragile” and sensitive to ATP depletion than cells of other types like osteosarcoma cells. In fact, we recently found that pancreata from patients with a mutation of mitochondrial DNA at the position of base pair 3243 showed a lower number of islet cells and exhibited their atrophy (predominantly β-cells), which may contribute to the pathogenesis of diabetes mellitus of such subjects.

In the current study, the [Ca\(^{2+}\)]i, was increased in the control cells by 20 mM glucose. This increase was lost in EB-treated cells and was restored after removal of EB. In this series of experiments, glucose-induced increase in [Ca\(^{2+}\)]i, was well associated with glucose responsiveness in insulin secretion. In contrast, 1 μM glibenclamide increased the [Ca\(^{2+}\)]i, in EB-treated cells to a similar extent as in control cells. These results indicate the involvement of mitochondrial function in the glucose-stimulated Ca\(^{2+}\) increase and subsequent insulin release, compatible with the current model that ATP generated from glucose in mitochondria is the key component in glucose-stimulated insulin release, acting upon ATP-sensitive K\(^{+}\) channels and subsequent activation of voltage-dependent calcium channels in β-cells (2, 39, 40).

The lack of response to glucose in EB-treated cells appears not to be due to the decreased expression of GK, which has been suggested to underlie the left shift of the dose-responsive curve of glucose-regulated insulin secretion in several β-cell lines (15, 41) and also in pancreatic islets (42). In EB-treated βHC9 cells, however, glucose-phosphorylating activities by HK or GK as

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2. S. Otabe and T. Kadowaki, unpublished observation.
well as expression levels of these enzymes were unchanged (Fig. 3). Regarding the glucokinase activity in this cell line, Liang et al. (16) reported that the V_max value of its activity of βHC9 cells was 10 times higher than that of hexokinase (in supernatant of homogenates), and the present study showed equal activities of these two enzymes (in sonicates). In this regard, it should be mentioned that the same laboratory (43) reported similar activities of glucokinase and hexokinase in sonicates of rat islets, whereas the former activity was less than half of the latter in the supernatant of the homogenates. This could be ascribed to the difference in the distribution between supernatant and sonicates of the two enzymes. In addition, 3-O-methyl-N-acetylglucosamine used to stabilize the assay by suppressing the perturbation by N-acetylglucosamine kinase (29, 43) might have affected glucokinase activity. In our hands, βHC9 cell glucokinase activity increases by 25% when measured without this compound.3

On day 6 when mitochondrial transcription was decreased to ~10%, the basal level of insulin secretion was enhanced (Fig. 5). This could be explained in part by the increase in insulin content as described earlier. It may also be due to decreased intercellular contact as a result of occasional loss of cells, for a decrease in cell-to-cell interaction has been proved in islets to cause elevation of the basal level of insulin secretion (44). This would agree well with the gradually decreasing basal secretion in the control cells along with the cell proliferation (Fig. 5A).

Other possibilities to be considered include the possibility that the basal level of [Ca^{2+}], might be increased (due to impaired activity of the Ca-ATPase pump, for example), which could contribute to the accelerated insulin secretion at low glucose levels; but, as shown in Fig. 9 and as described under “Results,” the basal [Ca^{2+}] level was lower in EB-treated cells than in control cells. Next, we considered that activation of the “immature” or constitutive secretory pathway with relative suppression of the regulatory pathway might be associated with the high basal secretion. Therefore, we measured the proinsulin/insulin ratio in the medium of the EB-treated and control cells on day 6 by high performance liquid chromatography analysis; the elevation of this ratio could be attributable to relative activation of constitutive secretion. The results showed no significant increase in the ratio for the cells deficient in mitochondrial function (data not shown), suggesting that impairment of the regulatory pathway is little or none in EB-treated cells. Third, this increase in basal secretion might also be due to defective energy production resulting from low (0.1 mM) glucose incubation; however, fractional secretions at glucose concentrations of 2.5 and 5.0 mM, known to maintain cellular energy production, were also raised in EB-treated cells compared with control cells (Fig. 6). Finally, the decreased mitochondrial volume in EB-treated cells (Fig. 4) may be associated with some changes in intracellular distribution of substances related to insulin secretion such as long-chain acyl-CoA (45), which is believed to be stored for the most part in mitochondria (46). In any case, both an enhanced level of insulin secretion and a loss of glucose responsiveness in EB-treated cells are correlated to mitochondrial dysfunction, since these characteristics were completely restored after EB removal. Thus, mitochondrial function might have some additional, unknown correlation to insulin secretion in β-cells other than its effects on ATP-sensitive K+ channels (47).

In conclusion, we have developed a unique system to study the role of mitochondrial function in insulin secretion. In our experience, EB rarely failed to exert an effect on insulin secretion. In this regard, this approach is both reproducible and reversible. These characteristics make the method useful for studying the mechanisms of insulin secretion, especially in

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3 I. Miwa, unpublished data.
relation to mitochondrial (dys)function. Our results presented here as well as the previous report (18) with a \( \rho^0 \) system in MIN6 cells demonstrate the necessity of mitochondrial function for glucose-stimulated insulin release. Our data clearly show that mitochondrial function is essential for glucose signaling for insulin secretion through generating an increase in [Ca\(^{2+}\)]\(_i\), with possible interplay with some other unknown mechanisms. Moreover, the present report is the first to demonstrate a reversible change, upon removal of the agent suppressing mitochondrial transduction. Our results show that 80–90% inhibition of transcription of mitochondrial DNA is enough to cause a similar change to \( \rho^0 \) cells in terms of glucose-stimulated insulin release and [Ca\(^{2+}\)]\(_i\) rise, which provides practical convenience and usefulness as an experimental system for mitochondrial dysfunction studies. It negates the need to maintain the \( \rho^0 \) cell line or to verify the complete absence of mitochondrial gene products. In addition, a variety of degrees of mitochondrial dysfunction can be induced by changing the duration of EB treatment. Finally, it should be pointed out that the system described here should also serve as a useful tool for investigating the role of mitochondrial function and dysfunction in cell lines of other origin.

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