Insulin secretion from glucose-stimulated pancreatic β-cells is oscillatory, and this is thought to result from oscillations in glucose metabolism. One of the primary metabolic stimulus-secretion coupling factors is the ATP/ADP ratio, which can oscillate as a result of oscillations in glycolysis. Using a novel multiwell culture plate system, we examined oscillations in insulin release and the ATP/ADP ratio in the clonal insulin-secreting cell lines HIT T-15 and INS-1. Insulin secretion from HIT cells grown in multiwell plates oscillated with a period of 4 min, similar to that seen previously in perfusion experiments. Oscillations in the ATP/ADP ratio in cells grown under the same conditions also occurred with a period of 4 min, as did oscillations in [Ca\textsuperscript{2+}], monitored by fluorescence microscopy. In INS-1 cells oscillations in insulin secretion, the ATP/ADP ratio, and [Ca\textsuperscript{2+}] were also seen, but with a shorter period of about 1.5 min. These observations of oscillations in the ATP/ADP ratio are consistent with their proposed role in driving the oscillations in [Ca\textsuperscript{2+}], and insulin secretion. Furthermore, these data show that, at least in the clonal β-cell lines, cell contact or even circulatory connection is not necessary for synchronous oscillations induced by a rise in glucose.

Insulin secretion from both perfused pancreas and perfused pancreatic islets has been shown to oscillate (1–3). These oscillations persist in dissociated islets and in clonal insulin-secreting cell lines, suggesting that the impetus for such oscillations is within the insulin-secreting cell (3, 4). The importance of oscillations in insulin release is highlighted by the fact that oscillations are often altered or impaired in patients with Type II diabetes (5). A widely accepted model of glucose-induced insulin release has been developed in which the driving force for oscillatory secretion is proposed to be oscillations in glucose metabolism (6–8). In this model glucose passes readily into the β-cell via the Glut-2 transporter to be phosphorylated by glucokinase, the first enzyme in the glycolytic pathway. Glucokinase has been described as the glucose sensor in the β-cell since it can regulate glucose entry into glycolysis over a physiologically relevant concentration range (9). Oscillations in glycolysis are a direct result of the allosteric regulation of phosphofructokinase (PFK).\textsuperscript{1} PFK is inhibited by ATP and activated by AMP and fructose 1,6-bisphosphate (F1,6bP), thus providing exquisite sensitivity to the energy state of the cell (10). The autocatalytic activation of PFK by its product F1,6bP results in bursts in the production of ATP from glycolysis and in oscillations of metabolites both up-and downstream of PFK (11). The oscillations in the ATP/ADP ratio thus produced would cause closing and reopening of ATP sensitive K\textsuperscript{+} (K\textsubscript{ATP}) channels (12), leading to oscillations in membrane potential and subsequent oscillations in voltage-gated Ca\textsuperscript{2+} channel activity (13). Oscillations in glucose-induced insulin secretion may result from oscillations both in the ATP/ADP ratio and cytoplasmic free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) (14).

There have been a number of studies showing a close association between membrane depolarization, [Ca\textsuperscript{2+}], changes, and insulin release (15, 16). Other experiments have demonstrated discordance between [Ca\textsuperscript{2+}], and insulin secretion when monitored simultaneously (17) or in parallel (18). When the regulation of Ca\textsuperscript{2+} influx is short circuited by the presence of KCl to depolarize the cell and diazoxide to maintain the KATP channel open and unresponsive to changes in the ATP/ADP ratio, glucose continues to cause an increase in secretion in a concentration-dependent manner (19). Secretion under these conditions (K\textsubscript{ATP}-channel-independent pathway) remains oscillatory while the [Ca\textsuperscript{2+}], is elevated and unchanging (3, 20). These data suggest that [Ca\textsuperscript{2+}], is not the sole regulator of insulin release in the pancreatic islet but rather may play a permissive role. Again using the K\textsubscript{ATP} channel-independent pathway to stimulate secretion, investigators found that formycin A, an adenosine analogue which was converted to formycin A-triphosphate in islets, was capable of potentiating mycin A, an adenosine analogue which was converted to formycin A-triphosphate in islets, was capable of potentiating

\textsuperscript{1} The abbreviations used are: PFK, phosphofructokinase; OOPSEG, Optimized Optimal Segments program; CV, coefficient of variance; K\textsubscript{ATP} channels, ATP-sensitive K\textsuperscript{+} channels; [Ca\textsuperscript{2+}], cytoplasmic free Ca\textsuperscript{2+} concentration; KRB, Krebs-Ringer bicarbonate buffer.

\textsuperscript{2} This work was supported by National Institutes of Health Grants DK35914 and DK53064, the Swedish Medical Research Council (03X-09890, 03XS-12708, 19X-00034), the Pharmacia & Upjohn Co., the Swedish Diabetes Association, the Berth von Kantzows Foundation, the Nordic Insulin Foundation Committee, and funds of the Karolinska Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

From the \#The Rolf Luft Center for Diabetes Research, Department of Molecular Medicine, Karolinska Institute, S-171 76 Stockholm, Sweden, the $Obesity Research Center, Evans Department of Medicine, Boston Medical Center, and the ¶Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118
ments, due to the inaccessibility of the cells either in columns or in microscope chambers. Measurements of oxygen consumption (22, 23) and NADH autofluorescence (24) have been correlated to both [Ca\(^{2+}\)] and insulin secretion. Oscillations in the ATP/ADP ratio have only been correlated to oscillations in [Ca\(^{2+}\)], under conditions in which the cells are stirred in a cuvette, preventing the simultaneous measurement of insulin secretion (25). Correlations between changes in the ATP/ADP ratio and K\(_{ATP}\) channel activity (26) and insulin secretion (27) have been made in clonal \(\beta\)-cells transfected with luciferase, but in these cases oscillations in the ATP/ADP ratio were not detected.

It is still not clear how the cells within an islet are coordinated or synchronized to each other to obtain oscillations in insulin release from single perfused islets (28). There is also a question as to how islets or cells with no cell-cell contact can be coordinated with each other to maintain an oscillatory pattern of secretion over time. It has been proposed that a yet unidentified factor secreted from the cells may play a role in maintaining metabolic synchrony, as is the case in yeast (3, 29).

We previously showed that HIT-T15 clonal insulin-secreting cells exhibit large oscillations in insulin secretion with a period of 15–20 min, the peaks of which were superimposed with smaller oscillations of 5–8 min period, when perfused in a column (3). The oscillations observed in these cells suggest that the populations of cells mixed with the beads were synchronous in their response to glucose. The synchronous response of these cells suggests that oscillations could also be evident in attached cultured cells in a multiwell plate. In this case, if a diffusible soluble factor controlled synchrony, oscillations with differing phases or periods might be obtained in the separate wells of the plate, thus masking the oscillations seen in the column.

By using a multiwell approach, we now show that HIT cells grown in a multiwell plate exhibit oscillations in insulin secretion with a period similar to that found in cells perfused in a column. Oscillations in insulin release were also observed from INS-1 cells similarly attached to multiwell plates. These oscillations in insulin release correlated with oscillations in the ATP/ADP ratio and are similar in period to those in [Ca\(^{2+}\)], confirming a role for metabolic oscillations in oscillatory insulin release.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Clonal insulin-secreting tumor cells, HIT-T15, were cultured in RPMI 1640 tissue culture medium supplemented with 50 units/ml penicillin, 50 \(\mu\)g/ml streptomycin, 32.5 \(\mu\)M glutathione, 0.1 \(\mu\)M selenous acid, and 10% fetal bovine serum. Cells were used between passages 65 and 100. INS-1 cells were cultured in RPMI 1640 medium supplemented with 50 units/ml penicillin, 50 \(\mu\)g/ml streptomycin, 50 \(\mu\)M 2-mercaptoethanol, 1 mM pyruvic acid, 10 mM HEPES, and 10% fetal bovine serum. Cells were used between passages 65 and 100.

**Insulin Secretion**—HIT cells grown in 48-well plates (Costar) were washed three times in Krebs-Ringer bicarbonate buffer (KRB) containing (in mM) 109 NaCl, 4.6 KCl, 5 NaHCO\(_3\), 2 CaCl\(_2\), 1 MgCl\(_2\), 1.15 NaHPO\(_4\), 0.4 KH\(_2\)PO\(_4\), 0.1 MgSO\(_4\), 5 glucose, and 20 HEPES, pH 7.4, and preincubated in the identical buffer for 30 min at 37 °C. The preincubation medium in each well was then replaced at 30 s intervals with KRB containing 10 mM glucose to produce a time course of insulin secretion. Thus, the first well was exposed to glucose for 24 min and the last for 30 s. Insulin was collected simultaneously from all the wells by inverting the test plate into a second 48-well plate. The cells were then quick frozen in liquid N\(_2\) and stored at −80 °C until extracted for ATP/ADP ratio measurements. The same procedure was followed for INS-1 cells, using a 10-s time interval for the addition of glucose to the wells over an 8-min time course. INS-1 cells were grown overnight in RPMI 1640 medium containing 3 mM glucose, preincubated for 30 min in KRB with 3 mM glucose and stimulated with 16.7 mM glucose. Insulin was measured by radioimmunoassay (Linco Research Inc., St. Louis, MO).

**Cell Extraction**—Cells were extracted in cold 1% (w/v) trichloroacetic acid (0.5 ml/well), and the trichloroacetic acid supernatant was neutralized with four equal volume washes of diethyl ether using vacuum suction to remove the ether between each wash. Neutralized supernatants were then freeze-dried in a Speed Vac (Savant) and stored at −80 °C until assayed for ATP and ADP.

**ATP/ADP Measurements**—Dried samples were resuspended in 0.5 ml of water and an aliquot was removed to measure ATP directly using luciferase coupled with a Cavro injector. ADP was converted to ATP by assayed luciferase after the sample was depleted of endogenous ATP with ATP sulfurylase, thus enhancing the sensitivity of the ATP measurement (30). Data are presented as the ATP/ADP ratio, making them independent of changes in cell number per well or loss of sample volume during extraction.

**Measurements of [Ca\(^{2+}\)]**, was measured in fura-2-loaded clonal insulin-secreting cells (31) grown on glass coverslips that were used as the bottom of a custom built perfusion chamber. The chamber was mounted on an inverted microscope (Zeiss Axiovor 135 TV, Zeiss, Germany) and excited at 340 nm and 380 nm using a SPEX fluorometer with the emission at 510 nm collected by a cooled CCD camera. Image analysis was performed with Inovision software (Durham, NC). Resting [Ca\(^{2+}\)] was measured in 0.1 mM glucose or 5 mM glutamine for HIT cells and 3 mM glucose for INS-1 cells. HIT cells were stimulated with 10 mM glucose plus 10 mM leucine, while INS-1 cells were stimulated with 20 mM glucose plus 10 mM leucine.

**Analysis of Oscillations**—Data were analyzed using the Bergman technique for estimating measurement error in time courses (32, 33). The program, Optimized Optimal Segments (OOPSEG), quantifies and filters random measurement error to reconstruct the error-free shape of data curves. This program iteratively assumes the error in the time-course data and fits the data to a line such that the residuals (the difference between the smoothed curve and data points) are random and consistent with the assumed error. Data are presented as smoothed curves, and the coefficient of variance (CV) is calculated. The derivative (slope) at each data point on the line is also estimated by the OOPSEG program and plotted. Periods of oscillations were calculated without the aid of a specific program using the derivative data from the OOPSEG program where applicable. Measurements of insulin release and the ATP/ADP ratio were not always made from the same cells. Statistical analysis was performed using Student’s t test for unpaired data.

**RESULTS**

HIT cells grown in 48-well plates and stimulated with 10 mM glucose, one well at a time at 30 s intervals, exhibited oscillations in insulin secretion (Fig. 1). Oscillations in the wells were seen as step jumps with increases in secretion (exocytosis turned on) followed by plateaus (exocytosis turned off) and are of a 4-min period, similar to those seen in the packed column. The secretion patterns from the wells were linearized by smoothing the data with a three point moving average, fitting it to a straight line (Fig. 1A), and then determining the positive or negative differences between the data and the straight line (residuals). Plotting the secretion from cells grown in multiwell plates as residuals clearly shows the oscillatory pattern of exocytosis (Fig. 1B).

Fig. 1C shows the same data unsmoothed and then smoothed using the OOPSEG analysis (32, 33). The derivative of the OOPSEG smoothed data is plotted in Fig. 1D and shows results similar in number of oscillations to the residual analysis (Fig. 1B). In the case of the OOPSEG derivative, the troughs (D) correlate to the plateaus (slope approaching zero) in insulin release (C) and are thus slightly right shifted from the peaks in 1B. These results indicate that cells from different wells with no possibility of contact exhibited synchronous oscillations in response to a step jump in glucose concentration.

The multiwell plate has the advantage of allowing access to the cells for metabolite measurements. The time course of changes in the ATP/ADP ratio, measured from 30 individual wells, is seen in Fig. 2. The smoothed ATP/ADP ratio determined by the OOPSEG program as well as the actual ATP/ADP ratio data is shown in Fig. 2A, while the derivative of the smoothed data is shown in Fig. 2B. The ATP/ADP ratio oscil-
The derivative of the OOPSEG smoothed data (in panel C (D and panels C) data were analyzed by OOPSEG in panels C and D). Unsmoothed data (open circles) are compared with the smooth curve obtained from OOPSEG analysis (closed circles) in panel C (CV = 7.3%). Panel D represents the derivative of the OOPSEG smoothed data (n = 3).

lated with a period similar to that of insulin secretion (Fig. 2, C and D).

[Ca\(^{2+}\)], determined by image analysis of fura-2-loaded HIT cells also oscillated in response to glucose stimulation with a period similar to that of the ATP/ADP ratio and insulin secretion (Fig. 2, E and F). Thirty-three of a total of 47 HIT cells examined in four separate experiments (70%) responded with an increased [Ca\(^{2+}\)], upon stimulation with glucose, with 16 cells (33%) exhibiting a clear oscillatory pattern. In the average signal from a field of cells the first [Ca\(^{2+}\)], oscillation was often much larger than those that followed due to the heterogeneity of the response after the initial increase in [Ca\(^{2+}\)], (data not shown). The average data showed that [Ca\(^{2+}\)], in the population oscillated with a similar period as the ATP/ADP ratio and insulin secretion (Table I).

In contrast, when another insulin-secreting cell line, INS-1, was exposed to stimulatory levels of glucose (20 mM), all of the cells responded with both an initial increase and oscillations in [Ca\(^{2+}\)], (49 cells in three experiments). These oscillations were faster than those observed in HIT cells, having an average period of 1.4 min (Fig. 3, E and F). When the oscillations in [Ca\(^{2+}\)], were averaged from 18 cells monitored in a single experiment, the oscillatory pattern was only slightly dampened compared with that obtained from a single cell (data not shown), indicating that all cells responded similarly. The average results show oscillations with a similar period to those found in the individual cells (Table I).

To determine whether the faster oscillations in [Ca\(^{2+}\)], found in INS-1 cells were due to faster oscillations in the ATP/ADP ratio in these cells, we again employed the multwell system. The ATP/ADP ratio in INS-1 cells was measured in response to raising the glucose concentration from 3 mM to 16.7 mM. Induced oscillations in the ATP/ADP ratio, when measured in the popula-

| Parameter       | HIT-T15       | INS-1       |
|-----------------|---------------|-------------|
| Period (min)    | 3.6 ± 0.3     | 1.4 ± 0.1*  |
|                  | (3)           | (3)         |
| [Ca\(^{2+}\)]   | 5.0 ± 0.5     | 1.4 ± 0.1*  |
|                  | (4)           | (3)         |
| Insulin release | 4.0 ± 0.5     | 1.5 ± 0.1*  |
|                  | (3)           | (5)         |

*p < 0.005 for oscillations in INS-1 cells compared to HIT-cells.

INS-1 cells grown in multiple wells and analyzed by OOPSEG (Fig. 3, A and B), were similar in period to the [Ca\(^{2+}\)], oscillations measured in the same cell line. Glucose-stimulated insulin secretion from INS-1 cells grown in the multwell plates and analyzed by OOPSEG (Fig. 3, C and D and Table I) had the same period as the ATP/ADP ratio and [Ca\(^{2+}\)]. Our data are
cells that were stimulated by glucose to act in unison. Presumably, the similar metabolic effects of glucose on the different groups of cells resulted in the apparent synchrony of the cells in separate wells. Admittedly, these oscillations were studied over a relatively short time (15 min), and it cannot be ruled out that a more active process of synchronization is involved in maintaining the oscillations that have been observed over much longer times (3, 22).

The advantage of the multiwell approach to study oscillatory secretion is the ability to quickly freeze-clamp the cells to examine related parameters. By using this approach, we demonstrated that oscillations in insulin secretion were similar to those in the ATP/ADP ratio and thus to metabolism. Changes in the ATP/ADP ratio occurred with the same period as that of secretion in HIT and INS-1 cells. This is the first time that oscillations in the ATP/ADP ratio have been shown to correlate with oscillations in insulin release in glucose-stimulated cells. Our data suggest that synchrony in secretion is determined by synchrony in glucose metabolism of the cells.

The glucose-induced oscillations in the ATP/ADP ratio and insulin release were also similar in period to the [Ca$^{2+}$], oscillations in both HIT cells and INS-1 cells. This, together with our previous demonstration that changes in the ATP/ADP ratio precede changes in [Ca$^{2+}$], (25, 35), is strong evidence that the metabolism of glucose, via changes in the ATP/ADP ratio, determines the level of [Ca$^{2+}$], maintained in the cell and which in turn drives exocytosis of insulin. The amplitude of oscillations in the ATP/ADP ratio was 2.4 ± 0.3 (n = 3) in the INS-1 cells compared with 1.7 ± 0.2 (n = 3) in the HIT cells. This may be the result of a larger proportion of glucose-responsive cells in the INS-1 cell population. Thus, the large number of nonresponsive HIT cells may dampen the changes observed in the ATP/ADP ratio in these cells. The lack of glucose response in many of the HIT cells was demonstrated by the inability of glucose to stimulate an increase in [Ca$^{2+}$], in 30% of the cells tested. Oscillations in [Ca$^{2+}$], occurred in almost all of the INS-1 cells tested, suggesting that the ATP/ADP ratio in all of these cells increased with glucose stimulation.

There is a striking difference in the period of oscillations in the ATP/ADP ratio, insulin secretion (as measured in the multiwells), and the [Ca$^{2+}$], changes between the two cell lines tested. Indeed, it is the contrasting nature of these two cell lines that highlights the importance of glucose metabolism in determining [Ca$^{2+}$], levels essential for secretion. The shorter oscillatory period in the ATP/ADP ratio of INS-1 cells may relate to increased flux through glycolysis due to differences in glucokinase expression (36) and/or differences in the isoform profile (37) or regulation of PFK (10), proposed to be the glycolytic oscillator in the β-cell. It is also possible that oscillations in other metabolites, enzymes, or channels may contribute to the differences in the pulsatility of insulin release between HIT cells and INS-1 cells.

**Acknowledgments**—We thank Drs. D. C. Bradley and R. N. Bergman for their kind gift of the Optimized Optimal Segments (OOPSEG) program used to analyze oscillations in the ATP/ADP ratio and insulin release.

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Glucose-induced Metabolic Oscillations Parallel Those of Ca$^{2+}$ and Insulin Release in Clonal Insulin-secreting Cells: A MULTIWELL APPROACH TO OSCILLATORY CELL BEHAVIOR
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J. Biol. Chem. 2001, 276:36946-36950.
doi: 10.1074/jbc.M105056200 originally published online July 31, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M105056200

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