Rhythm of the β-cell oscillator is not governed by a single regulator: multiple systems contribute to oscillatory behavior

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Heart E, Smith PJS. Rhythm of the β-cell oscillator is not governed by a single regulator: multiple systems contribute to oscillatory behavior. *Am J Physiol Endocrinol Metab* 292: E1295–E1300, 2007. First published January 9, 2007; doi:10.1152/ajpendo.00648.2006.—Pulsatile insulin output, paralleled by oscillations in intracellular Ca^{2+}, reflect oscillating metabolism within β-cells in response to secretory fuels. Here we question whether oscillatory periodicity is conserved or varied from stimulation to stimulation, whether glycolysis is essential for the manifestation of an oscillatory response, and if an environment of nutrient oversupply affects oscillatory regularity. We have determined that a β-cell oscillatory Ca^{2+} pattern is independent of the type of applied secretory fuel (glucose, methyl-pyruvate, or α-ketoisocaproate). In addition, single cells respond with the same pattern when repeatedly stimulated, regardless of the type of stimulatory fuel. Presence of substimulatory glucose is not necessary to obtain an oscillatory responses to methyl-pyruvate or α-ketoisocaproate. Glucose-6-phosphate, as a measure of glycolytic flux, is not detectable under these conditions. These data suggest that multiple systems, rather than a single enzyme component, can contribute to the β-cell oscillatory behavior. Prolonged exposure to high levels of palmitate impaired oscillatory regularity in the individual β-cells. This supports the hypothesis that a high-fat environment might contribute to loss of regular oscillatory pattern in diabetic subjects, acting, at least in part, at the level of the single β-cell.

insulin secretion; secretory fuels; Ca^{2+} oscillations

INSULIN RELEASE FROM THE PANCREAS is pulsatile (11, 20). The regular oscillatory pattern of insulin secretion, in contrast to a steady-state infusion, is important for a more functional hormone interaction with the peripheral tissues (32, 35) and has been reported to be impaired in Type 2 diabetic patients (1). The single β-cell is a metabolic oscillator, and it is widely accepted that, under in vitro conditions, such as isolated islets or single β-cells, oscillatory insulin secretion is a consequence of oscillations in cellular metabolism (19). Oscillation in β-cell metabolism leads to oscillations in the ATP-to-ADP ratio, which then drives oscillation in the activity of the ATP-sensitive K^+ channels, leading to plasma membrane depolarization, Ca^{2+} influx, and insulin output (15). Oscillations in a variety of parameters, such as oxygen consumption, mitochondrial membrane potential, ATP-to-ADP ratio, and Ca^{2+} activity, have been reported in single clonal or primary β-cells (19), but what systems are responsible for the generation of these oscillations, as well as how these oscillations are affected by the nutrient environment, has not been clearly addressed. While individual β-cells have been shown to display different sensitivities to glucose (6), the issue of how an individual cell responds to repetitive fuel stimuli is not known.

Oscillatory glycolysis and the action of its proposed pacemaker, the allosteric enzyme phosphofructokinase (PFK), has been reported to take place in β-cells. It represents a single-component model, according to which β-cell metabolic oscillations are an obligatory consequence of oscillatory glycolysis, governed by PFK (17, 43). We have tested this hypothesis by using nonglycogenic secretagogues in the absence of substimulatory glucose and measured levels of glycolytic flux under these conditions. We have found that β-cells display oscillations under conditions where nonglycogenic fuels are employed and glycolytic flux is absent. Furthermore, we have shown that a conserved periodicity is independent of fuel type. We have also determined how an environment of nutrient oversupply affects β-cell oscillatory behavior. Our study suggests that oscillatory metabolism is potentially underwritten by multiple components and is susceptible to modulation by the nutrient environment.

METHODS

Animals. Male Swiss-Webster mice (Charles River) were euthanized by halothane. All procedures were performed in accordance with the Institutional Guidelines for Animal Care, in compliance with United States Public Health Service regulations.

Islet isolation and primary cell culture. Pancreatic islets were isolated by collagenase (Roche) digestion, as previously described (13). Following overnight culture, islets were dispersed by incubation in Ca^{2+}/Mg^{2+}-free PBS containing 3 mM EGTA and 0.002% trypsin for 10 min at 37°C with occasional agitation. Cells were centrifuged, washed, suspended in RPMI-1640 culture media (Sigma) supplemented with 5 mM glucose, 10% fetal calf serum (HyClone), 100 IU/ml penicillin, and 100 μg/ml streptomycin, and plated on poly-D-lysine-coated coverslips (MatTek, Ashland, MA) in 35-mm petri dishes (Ca^{2+} studies) or 48-well plates (secretion).

Ca^{2+} measurement. Single mouse islet cells were cultured for 72 h in the absence (control) or presence of 0.4 mM palmitate (lipotoxicity). Cells were loaded for 30 min with Ca^{2+} indicator fluo-4-AM (Molecular Probes) in the growth media, washed in Krebs-Ringer bicarbonate (KRB) buffer (4 mM glucose, 140 mM NaCl, 30 mM HEPES, 4.6. mM KCl, 1 mM $MgSO_4$, 0.15 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 5 mM $NaHCO_3$, 2 mM $CaCl_2$ and 0.05% BSA, pH 7.4, osmolarity 284 mosM), allowed to equilibrate for 15 min, and imaged on Zeiss-510 confocal microscope equipped with a heated stage. Fluo-4 was excited using 488 line of argon laser. Images were taken every 10 s. Both control and palmitate-treated cells were used for a single-step stimulation protocol with 10 mM glucose to determine Ca^{2+} oscillatory regularity, which was calculated as standard deviation (SD) from the mean oscillatory period (OP) for that particular trace. Control cells were used for experimental series as follows. In the three-step protocol of *series 1*, glucose was raised from 4 mM to stimulatory 10 mM (*step 1*), lowered to 0 mM (*step 2*), and raised to

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10 mM (*step 3*). [pH of the KRB buffer was not changed in the presence of 10 mM methyl-pyruvate (MeP) or α-ketoisocaproate (KIC) for the duration of the experiment.] In *series 2* and *3*, MeP (10 mM) or KIC (10 mM), respectively, were applied in the absence of any glucose, in *step 3*. Scanning was paused in *step 2* to minimize photodamage to the cells. Images were analyzed by the LSM Image Browser software to derive Ca^{2+} profiles. After the experiment, dishes were fixed in paraformaldehyde, and β-cells were identified using guinea pig anti-insulin antibody (Zymed, San Francisco, CA). Only insulin-positive (β-cells) were included in the data analysis.

Insulin secretion. The amount of released insulin was determined after 30 min of static incubation with a radioimmunoassay kit (Linco Research, St. Charles, MO) using rat insulin as the standard.

Glucose 6-phosphate levels. Glucose 6-phosphate (G-6-*P*) levels were determined in TCA neutralized islet extracts by the cycling method of Lowry (22). Fifty to one-hundred islets per sample were used.

Materials. Unless otherwise indicated, all reagents were obtained from Sigma-Aldrich (St. Louis, MO).

RESULTS

The absence of substimulatory glucose during stimulation with nonglycogenic fuels MeP and KIC did not affect levels of insulin secretion (Fig. 1). Since it is established that the Ca^{2+} response and insulin output occur in parallel (15), we expected that the level of Ca^{2+} would not be affected by the absence of nonstimulatory glucose, and, therefore, we evaluated its oscillatory character under the same stimulatory conditions.

For Ca^{2+} studies, single cells were prepared from islets by dissociation and were subjected to the stimulation protocol that allowed us to determine how individual cells would respond to the repeated exposure of the same fuel (glucose), as well as second exposure to different fuels (KIC, MeP) in the absence of substimulatory glucose. The phenomenon of whether an individual cell will respond in the same or different manner to the repeated fuel challenge [a feature sometimes called "metabolic memory" (3)] has not been investigated before in β-cells. Individual β-cells responded to stimulatory fuel addition with a wide variety of Ca^{2+} OP and lags (L), with average OP and L equal to 4.4 ± 1.7 and 4.1 ± 1.4 min, respectively. In individual β-cells, however, the second response was similar to the first (Fig. 2), as determined by comparison of the L and OP of the first and second Ca^{2+} response in the same cells for all fuel types ($L_2 = 121 \pm 14\%$ of L_1, $OP_2 = 94 \pm 15\%$ of
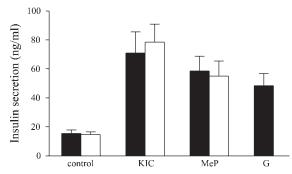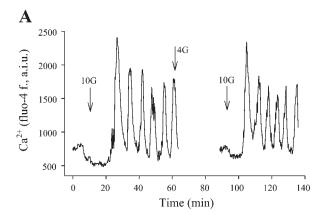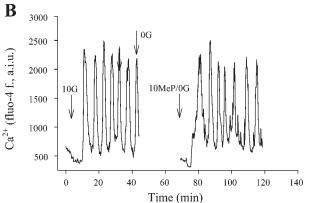
Fig. 1. Effect of substimulatory glucose on α-ketoisocaproate (KIC)- and methyl-pyruvate (MeP)-dependent secretion. Mouse islets (10 islets/sample) were incubated for 30 min, either without secretory fuels (control) or with 10 mM KIC, MeP, or glucose (G), in the presence (solid bar) or absence (open bar) of 4 mM glucose. Data are average of 3 independent experiments, expressed as means ± SE.

Fig. 2. *A*, *B*, and *C*: examples of single β-cell Ca^{2+} responses to sequential challenge with stimulatory fuels G (*n* = 33), MeP (*n* = 29), and KIC (*n* = 26), where *n* represents number of cells. Cells were challenged with 3-step stimulation protocol, as described in METHODS. Additions of fuels are indicated by arrows. All cells examined behaved in a similar manner to these examples. aiu, Arbitrary intensity unit.

OP_1, *n* = 78). The response is thus conserved within an individual cell. This is further illustrated in Fig. 3, where the differences of OP_2-OP_1 are smaller when both OPs are calculated from an individual cell (*A*), compared with the large values of OP_2-OP_1 when OP_2 is calculated as an average from the rest of the cell population (*B*). This effect occurred, regardless of the type of secondary fuel, as is apparent from Fig. 2, *B* and *C*, which also illustrate that the absence of substimulatory (4 mM) glucose did not prevent oscillations following the application of the nonglycogenic fuels MeP and KIC. In these experiments, after recordings were obtained in the presence of stimulatory 10 mM glucose, cells were washed three times in

A

B

Fig. 3. Oscillatory responses in single cells are conserved and fuel-type independent. Differences are shown between the first (OP_1) and second oscillatory periods (OP_2), where both OP_1 and OP_2 are calculated from the same individual cell (A; ●) or where OP_2 is calculated as an average from the rest of cells (B; ○); $n = 78$.

KRB, with no glucose, and allowed to downregulate in this buffer for 30 min before the application of the stimulatory concentration of the second fuel. Nonstimulatory levels of both fuels (1 mM) were included during this period, providing cells with a carbon source. To ascertain 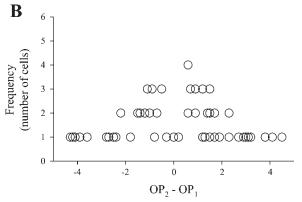that, under these experimental conditions, glycolysis did not take place, G-6-P levels, as a measure of glycolytic flux, were determined in islets maintained for 30 min in 0, 4, and 10 mM glucose in the presence or absence of 10 mM MeP or KIC, by the Lowry cycling method. Data presented in Table 1 demonstrate that no glycolytic flux was detected when islets were maintained in the absence of extracellular glucose. This is consistent with the fact that islets lack gluconeogenic phosphoenolpyruvate car-

Table 1. *G-6-P levels in mouse islets*

Glucose, mM		MeP	KIC
0	ND	ND	ND
4	0.55±0.06	0.75±0.09	0.62±0.04
10	1.56±0.25	1.64±0.35	1.46±0.28
20	2.6±0.34	2.52±0.34	2.83±0.18

Values, presented as pmol glucose 6-phosphate (G-6-P)/islet, are means ± SE from 3 independent experiments. Islets (50) were exposed for 30 min to various glucose concentrations (0–20 mM) in the presence or absence of 10 mM methyl-pyruvate (MeP) or α-ketoisocaproate (KIC). G-6-P levels were measured in islet extracts as described in METHODS. ND, not detected.

boxykinase and, therefore, would not be able to synthesize glucose (26, 29).

Next, we examined how oscillatory regularity is affected under a lipotoxic condition, which was induced by incubating the cells in the 0.4 mM palmitate for 72 h. Cells were then downregulated for 3 h in the normal medium, and the stimulatory protocol included a single-step jump from 4 to 10 mM glucose. Palmitate exposure resulted in a decrease in the number of cells exhibiting oscillatory response (56 vs. 73% in control group), as well as a decreasing regularity of the response in those cells that retained a capacity to oscillate. Examples of a regular vs. irregular oscillatory response are presented in Fig. 4, *A* and *B*. Regularity was calculated as the

A

B

C

Fig. 4. Effect of palmitate exposure on oscillatory regularity in individual β-cells. Cells were cultured in the absence (example in *A*, ● in *C*, $n = 56$), or presence of 0.4 mM palmitate (example in *B*, ○ in *C*, $n = 60$) for 72 h. *C*: oscillatory regularity is expressed as standard deviation (SD) from the average oscillatory period calculated for each cell.

standard deviation from the average OP. Figure 4*C* illustrates that regularity of fuel-induced oscillations was impaired after cell culture in palmitate, compared with the control.

DISCUSSION

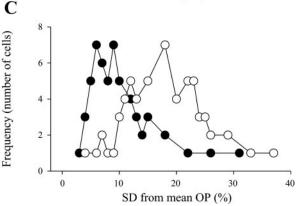We have shown for the first time that individual β-cell oscillatory response to stimulatory levels of fuels is conserved and fuel-type independent within a single β-cell and, therefore, likely reflects a metabolic setting characteristic for the cell. We have further shown that these oscillations can occur via glycolysis-independent pathways, and that their oscillatory regularity is affected by saturated fatty acids.

Factors that drive and regulate oscillations in the β-cell metabolism remain under investigation. A model of glycolytic oscillations, as a driving force behind β-cell metabolic oscillations and ultimately insulin secretion, has been proposed (17, 43). According to this model, the allosteric enzyme PFK (PFK$_m$, muscle type) is proposed to be the sole pacemaker of metabolic oscillations in the β-cell (44) and is under the regulatory control of a variety of substrates, including citrate and ATP. This forms a negative feedback loop, which generates pulsatile delivery of the glycolytic product pyruvate to the TCA cycle, resulting in oscillation of oxygen consumption and the ATP-to-ADP ratio (4). Oscillations in the ATP-to-ADP ratio then cause oscillations in the activity of the ATP-sensitive K$^+$ channels, which leads to the oscillatory Ca^{2+} influx and insulin secretion (15). Studies that report impairment of oscillation in subjects with a deficiency in PFK$_m$ seem to support a role for this enzyme (38). However, in the latter study, only two subjects were evaluated, and, although observed patterns were irregular, oscillations were still apparent. It is not known at present what residual amount of PFK is sufficient to drive glycolytic oscillations. Taking into account that this enzyme is relatively abundant, only a fraction might be sufficient to contribute to the maintenance of the oscillatory glycolysis. This issue is complicated by the fact that disappearance of the oscillatory response in β-cells, from islet-specific PFK$_m$ knockout model, has not been demonstrated. As a consequence of the singular importance given to the role of PFK, the above model must assume that all other metabolic downstream processes are nonoscillatory. This is unlikely, as shown in reports describing oscillations in citrate (28) and in mitochondrial enzymes (25) in isolated β-cell mitochondrial preparations, a system devoid of glycolysis, as well as oscillations in β-cell metabolism in response to hormone stimulation (7, 12). In mathematical models, other factors besides glycolysis, such as ionic fluxes (8, 9, 34), have been proposed to play an important role in the oscillatory response. The hypothesis that the oscillatory pattern is a result of multicomponent phenomena, and not just under the control of one single enzyme, has been proposed elsewhere (36, 42) and is consistent with the fact that all nonlinear systems operating far away from the equilibrium, such as enzymatic reactions, display oscillations (10, 18). In yeast, where oscillations in glycolysis were first described (2), it has been suggested that glycolytic control, confined solely to PFK, is not sufficient to account for metabolic oscillations (37) and that control of oscillations is distributed over a number of molecular processes (41).

In several studies, the effect of various secretagogues, with and without the presence of substimulatory glucose, on secre-

tion and oscillatory metabolism has been investigated, and reported results vary greatly. In some studies, KIC, MeP, glyceraldehyde, and dihydroxyacetone were reported to be unable to elicit Ca^{2+} oscillations alone (17) or with glucose present (5, 21). In other reports, KIC induced slow Ca^{2+} oscillations (31) and secretion in the absence of substimulatory glucose (14, 40), which agrees with our data. In some of these studies, the nonglycogenic substrate (MeP) was applied in a concentration (20 mM) (5) that has been shown to block secretion (30), making it difficult to interpret such data. Some secretagogues, such as glyceraldehyde, have been shown to be toxic and not metabolized via the glycolytic pathway to an extent proposed earlier (27), and fuels such as pyruvate or succinate are not membrane permeable and, as such, would not stimulate metabolism in contrast to their methylated analogs (24). In many of these studies, secretion was not measured in parallel to ensure that the fuel concentration used was indeed stimulatory.

The character of the oscillatory response to stimulatory fuels in whole islets, as well as in single-cell preparations, may depend on the quality of the isolation procedure, as a consequence of which some cells or islets will establish oscillations, whereas others will not. Indeed, dispersion of the islets into the single cells results in >50% cell death (16) and likely affects the integrity of the plasma membrane of some living β-cells, a feature crucial for demonstrating oscillations. It is, therefore, of a critical importance to establish that a cell or islet retained its capacity to respond to fuels in an oscillatory manner, before making a conclusion whether a particular fuel can produce oscillatory response. Only in a few studies have the same cells or islets been challenged sequentially with glucose and nonglycogenic fuel, making sure that such a system has the capacity to oscillate.

It might be argued that, in the absence of extracellular glucose, additional glucose can be provided to the β-cells through glycogenolysis. Thus the glycogen-derived glucose could enable glycolytic flux via PFK. It is unknown, however, what would be the time frame of glycogen mobilization, if this process indeed takes place. To exclude this possibility, and prove beyond a reasonable doubt that glycolytic flux was absent under conditions when MeP or KIC were used, G-6-*P* levels were measured in the whole islets under the same conditions used for Ca^{2+} studies. While levels of G-6-*P* in cells cultured in the presence of stimulatory (10 mM) or substimulatory (4 mM) glucose were detectable, and in general agreement with the data reported before (33), we did not detect G-6-*P* in the samples cultured in the absence of glucose. This confirms that, under these conditions, glycolytic flux is below detectable levels and, therefore, unlikely to influence metabolism.

Our data suggest that, although it is an indisputable fact that oscillatory glycolysis has a role and contributes to the generation of the metabolic oscillations when stimulatory glucose is applied, there are alternatives to drive the β-cell oscillations. Providing nonglycogenic fuels in the absence of substimulatory glucose directly demonstrates that an oscillatory capacity is preserved and conserved under the conditions when glycolytic flux is absent. This implies that the determinants of oscillatory frequency are downstream of these metabolic effectors.

Impairment of the regular oscillatory output has been described in Type 2 diabetic subjects (1), and the high-fat diet,

resulting in the increased level of circulating saturated fatty acids, has been implicated in the development of Type 2 diabetes (23). It has been established that prolonged exposure to high levels of elevated fatty acids (a condition termed lipotoxicity) decreases glucose-stimulated insulin secretion in isolated islets (39). How lipotoxicity affects the oscillatory character of the response has not been addressed. The final oscillatory profile likely reflects a hierarchy involving *1*) single β-cell oscillator, *2*) synchronization of the single β-cells inside an islet, and *3*) synchronization of islets, or at least population of islets, through the pancreas, which happens most likely via the nervous system. Although it is possible that all or at least one level must be involved, we investigated single β-cell oscillatory response after 72 h of palmitate culture. Palmitate exposure impaired regularity of the oscillatory response in these cells. This suggests that high-fat diet contributes to a loss of oscillatory regularity, at least at the single-cell level.

Altogether, our data show that the generation in β-cell metabolic oscillations is potentially a result of multiple factors and is affected by the nutrient environment.

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