Tissue-dependent loss of phosphofructokinase-M in mice with interrupted activity of the distal promoter: impairment in insulin secretion

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Richard A-M, Webb D-L, Goodman JM, Schultz V, Flanagan JN, Getty-Kaushik L, Deeney JT, Yaney GC, Dunaway GA, Berggren P-O, Tornheim K. Tissue-dependent loss of phosphofructokinase-M in mice with interrupted activity of the distal promoter: impairment in insulin secretion. Am J Physiol Endocrinol Metab 293: E794–E801, 2007. First published June 26, 2007; doi:10.1152/ajpendo.00168.2007.—Phosphofructokinase is a key enzyme of glycolysis that exists as homo- and heterotetramers of three subunit isoforms: muscle, liver, and C type. Mice with a disrupting tag inserted near the distal promoter of the phosphofructokinase-M gene showed tissue-dependent differences in loss of that isoform: 99% in brain and 95–98% in islets, but only 50–75% in skeletal muscle and little if any loss in heart. This correlated with the continued presence of proximal transcripts specifically in muscle tissues. These data strongly support the proposed two-promoter system of the gene, with ubiquitous use of the distal promoter and additional use of the proximal promoter selectively in muscle. Interestingly, the mice were glucose intolerant and had somewhat elevated fasting and fed blood glucose levels; however, they did not have an abnormal insulin tolerance test, consistent with the less pronounced loss of phosphofructokinase-M in muscle. Isolated perifused islets showed about 50% decreased glucose-stimulated insulin secretion and reduced amplitude and regularity of secretory oscillations. Oscillations in cytoplasmic free Ca2+ and the rise in the ATP/ADP ratio appeared normal. Secretory oscillations still occurred in the presence of diazoxide and high KCl, indicating an oscillation mechanism not requiring dynamic Ca2+ changes. The results suggest the importance of phosphofructokinase-M for insulin secretion, although glucokinase is the overall rate-limiting glucose sensor. Whether the Ca2+ response to glucose in isolated islets was impaired. Glucose stimulation of insulin secretion requires glucose metabolism and involves a rise in the ATP/ADP ratio to close ATP-sensitive K+ channels and trigger Ca2+ influx. Furthermore, normally there are oscillations in muscle metabolism, cytoplasmic free Ca2+ concentration ([Ca2+]i), and insulin secretion, and we have proposed that these may be due to underlying oscillations in glycolysis generated by PFK-M (9, 44). However, [Ca2+]i, and secretory oscillations were still seen in PFK-M-deficient islets/cells. This may indicate that there is another underlying oscillation mechanism; alternatively, because of the great excess of PFK over glucokinase in islets, the 2–5% residual PFK-M seen in the PFK-M-deficient islets may still have been sufficient to generate the oscillations.

MATERIALS AND METHODS

Animals. Heterozygous mice with a disrupting tag inserted in the noncoding region of the PFK-M gene (OmniBank OST no. 56064, 50% C57BL/6 albino, 50% 129svEvBr) were received initially from Lexicon Genetics (The Woodlands, TX). OmniBank is a library of more than 200,000 mouse embryonic stem cell clones, each containing a gene trap insertion (created by insertional mutagenesis) in a single gene (55). OST no. 56064 was the only match to the PFK-M
gene. The mice were bred in-house at Boston University Medical Center to generate wild-type and homozygous PFK-M-deficient mice. (Note: “PFK-M” has previously been used to refer to the homotetramer M₄ or muscle PFK, especially in kinetic studies. In this paper, “PFK-M deficient” technically refers to loss of the subunit isoform as detected by Western analysis; this implies loss of M₄ but also of M-containing heterotrimeric in tissues expressing other isoforms.) Mice were used from breeder colonies to avoid any effects of maternal hyperglycemia, since mild hyperglycemia was noted in homozygous females (see RESULTS). Genotyping (nptII Invader Assay; Third Wave Technologies) of the initial 220 offspring yielded results corresponding with Mendelian inheritance: 25% wild type, 55% heterozygotes, and 20% homozygotes, indicating that the mutation was not acutely detrimental to development. Most litters consisted of 5–8 pups, and there were no deaths among newborns. Mice used for experiments were 3–6 mo of age; no consistent difference in weight was observed between wild-type and homozygous mice of this age. All procedures were approved by the Boston University Medical Center Animal Care and Use Committee.

Intraperitoneal glucose tolerance test. Blood glucose from the tail vein of overnight-fasted female mice was measured with a portable glucometer before and after glucose injection (1 mg/g body wt). For insulin radioimmunoassay, blood was collected in heparinized tubes, placed on ice, centrifuged, and stored at −20°C.

Intraperitoneal insulin tolerance test. Blood glucose levels were measured in fed female mice before and after insulin injection (0.75 U/kg body wt).

Tissue extraction for Western analysis and PFK activity. Tissues (gastrocnemius or quadriceps muscle, heart, and brain) were frozen in liquid nitrogen and extracted as described previously in 6 volumes of extraction buffer (54). Samples were fast-frozen in liquid nitrogen and stored at −80°C.

Western analysis of PFK subunit isoforms. PFK subunit isoforms were separated using SDS-PAGE and blotted essentially as previously described (54). The primary polyclonal antibodies were rabbit anti-dog muscle PFK, which recognizes only the M isoform and rabbit anti-rat brain PFK raised against all three isoforms (27).

PFK activity measurements. PFK activity stimulated by F16BP (20 μM) under inhibitory conditions (0.1 mM fructose 6-phosphate, 2 mM ATP, 0.02 mM AMP, pH 7) was measured in the high-speed supernatant fraction from mouse tissue extracts as described previously (54). Maximal PFK activity was assayed by measuring F16BP production in the presence of 2 mM fructose 6-phosphate and 1 mM ATP, pH 8.2 (2).

Real-time PCR. Primers were designed using Primer Express v2.0 software (PerkinElmer Applied Biosystems, Foster City, CA) to ensure suitability for the ABI Prism 7000 sequence detection system used and the reaction parameters according to the manufacturer’s protocol. Three sets of primers were designed, one for total PFK-M and one for the distal amplicon to detect loss of the M type subunit in homozygous animals, which is characteristic of PFK-M, was decreased 50%, but the activity stimulated by F16BP, which is limited by background ATPase activity).

RESULTS

PFK expression in mouse tissues. There was tissue-dependent loss of the M-type subunit in homozygous animals, which we therefore refer to as PFK-M deficient. Thus, Western analysis using a PFK-M-specific antibody showed 50–75% loss in skeletal muscle and little change in heart but 90% loss in brain (Fig. 1, A and B). Enzymatic activity measured spectrophotometrically in some samples showed similar results. In brain, which normally has ~50% M-type subunit (data not shown) (14, 15, 54), the maximal total activity was decreased 50%, but the activity stimulated by F16BP, which is characteristic of PFK-M, was decreased >95% (sensitivity limited by background ATPase activity).

The likely explanation for the large tissue-dependent differences in loss of PFK-M is disruption of the distal promoter. The distal transcription start site is normally used ubiquitously, whereas the proximal transcription initiation site was reported to be used primarily in muscle in mice (9). In agreement with this, skeletal muscle, heart, and brain from wild-type mice all contained distal promoter transcript; skeletal muscle and heart also had detectable levels of proximal promoter transcript, whereas the brain did not (Table 1). Levels of the distal promoter transcript were greatly decreased (>99%) in all of these tissues from the PFK-M-deficient mice. Levels of the proximal promoter transcript were decreased somewhat (50%)
in skeletal muscle and increased in heart and remained essentially undetectable in brain. Total PFK-M transcript levels were decreased perhaps 30–40% in skeletal muscle and heart but 99% in brain.

In initial Western blotting studies in pancreatic islets from one or two PFK-M-deficient mice, the M-type subunit could not be detected (>85–90% reduction). To better quantitate the

### Table 1. Expression of PFK-M transcripts in tissues from wild-type and PFK-M-deficient mice

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Wild Type</th>
<th>PFK-M Deficient</th>
</tr>
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<tbody>
<tr>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>100 (C&lt;sub&gt;T&lt;/sub&gt; 19.3)</td>
<td>61</td>
</tr>
<tr>
<td>Heart</td>
<td>10.5</td>
<td>7.4</td>
</tr>
<tr>
<td>Brain</td>
<td>5.1</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Distal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>100 (C&lt;sub&gt;T&lt;/sub&gt; 25.7)</td>
<td>0.14</td>
</tr>
<tr>
<td>Heart</td>
<td>39</td>
<td>&lt;0.07</td>
</tr>
<tr>
<td>Brain</td>
<td>22</td>
<td>&lt;0.07</td>
</tr>
<tr>
<td>Proximal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>100 (C&lt;sub&gt;T&lt;/sub&gt; 24.4)</td>
<td>50</td>
</tr>
<tr>
<td>Heart</td>
<td>1.0</td>
<td>8.2</td>
</tr>
<tr>
<td>Brain</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>

Data are presented as % value for wild-type muscle for each transcript; total message, distal transcript, proximal transcript. PFK-M, phosphofructokinase-M; C<sub>T</sub>, threshold cycle. Data were calculated using the ΔΔC<sub>T</sub> method to determine relative gene expression according to User Bulletin no. 2: Rev B-Relative Quantitation of Gene Expression (Applied Biosystems).

In vivo measurements: glucose and insulin tolerance tests. Female PFK-M-deficient mice had elevated fasting blood glucose levels [7.6 ± 0.3 mM (means ± SE), n = 6, vs. 4.6 ± 0.6 mM, n = 3, in wild-type controls; P = 0.001] and exhibited impaired glucose tolerance (Fig. 2). Basal insulin values were not significantly different (0.44 ± 0.07 vs. 0.34 ± 0.08 ng/ml, PFK-M deficient vs. wild type, n = 4 and 5, respectively). Glucose-stimulated insulin levels (15 min after injection) were not significantly lower in the PFK-M-deficient mice (0.64 ± 0.14 vs. 0.78 ± 0.16 ng/ml in wild type), but the percentage increase in insulin levels was significantly lower in the PFK-M-deficient mice (42 ± 15 vs. 140 ± 32% in wild type, P < 0.02). Insulin tolerance tests on fed female mice showed a similar drop in blood glucose in response to the insulin in the two groups (Fig. 3), indicating that the PFK-M-deficient mice were not grossly insulin resistant. Basal blood glucose levels were also elevated in these nonfasted animals (11.2 ± 0.3 mM, n = 5, vs. 8.2 ± 0.2 mM, n = 7, for wild type; P < 0.0001). The absence of insulin resistance argues that the glucose intolerance is due largely to a defect in insulin secretion.

**Insulin secretion from isolated islets.** Islets isolated from wild-type and PFK-M-deficient mice were perfused with glucose in a column. Although both groups of islets responded to stimulatory concentrations of glucose (11 or 16.7 mM), there was ~50% reduction in average secretion from the PFK-M-deficient islets compared with wild type (Fig. 4 and Table 2). The amplitude of the insulin pulses was also reduced in PFK-M-deficient islets, whereas the period remained unchanged. Assay of the total insulin content in the islets retrieved from the columns of the experiment shown in Fig. 4C showed that the PFK-M-deficient group did not have less insulin (in fact, it had ~20% more). Therefore, the secretary
defect is not due to insulin deficiency. The regularity of pulses was analyzed by measurement of approximate entropy for each group, where a relatively lower value indicates greater regularity (36). In general, pulsatile secretion from PFK-M-deficient islets was more irregular than that from wild type (approximate entropy = 0.002 ± 0.000 in PFK-M-deficient group vs. 0.0003 ± 0.0001 in wild type, n = 4; P = 0.00002). Basal (3 mM glucose) secretion was very low compared with stimulated secretion, and there was no consistent difference in basal secretion between PFK-M-deficient and wild-type islets.

[Ca^{2+}]_i oscillations in islet cells. Slow oscillations in [Ca^{2+}], normally correlate with insulin oscillations (2, 18) and can be measured at the single-cell level. Oscillations in [Ca^{2+}], in response to glucose stimulation were observed in cell clusters from PFK-M-deficient islets (Fig. 5) in a proportion of cells similar to that of wild-type cells. Thus, of the 139 cells from three PFK-M-deficient mice, 91% responded to 8 mM glucose and 71% exhibited oscillations, whereas of the 111 cells from three wild-type mice, 92% responded to 8 mM glucose and 75% exhibited oscillations. To test the possibility that a small percentage of cells from PFK-M-deficient islets might not be deficient (corresponding to the residual 2–5% PFK-M seen in Western analysis) and might thus be serving as pacemakers in cell clusters, we also examined single cells not in clusters; however, 50% (of 10 cells) were still seen to oscillate (compared with 7 of 10 wild-type cells). Basal [Ca^{2+}], and initial and second-phase glucose-stimulated average [Ca^{2+}], appeared to be similar. The time of onset of the Ca^{2+} response after glucose addition was also not different (98 ± 5 s, n = 112, for PFK-M deficient vs. 110 ± 7 s, n = 87, for wild type).

ATP/ADP ratio. The rise in the ATP/ADP ratio in response to glucose stimulation was normal in PFK-M-deficient islets. Thus the ATP/ADP ratio was 2.5 ± 0.1 at 3 mM glucose and 4.5 ± 0.2 and 4.4 ± 0.3 after 1 and 5 min, respectively, of 11 mM glucose in PFK-M-deficient islets, compared with a rise from 2.5 ± 0.1 to 4.5 ± 0.2 and 4.5 ± 0.1 in wild-type islets, combining the data of two experiments each with 3–4 samples of islets per data point. In a third experiment with somewhat lower values, there was also no difference between PFK-M-deficient and wild-type islets.

Insulin oscillations in the presence of diazoxide and high KCl. Alternative mechanisms have been suggested for oscillations in [Ca^{2+}], and, hence, insulin secretion that involve Ca^{2+} feedback effects on K_{ATP} channels (42). If this occurs, it could hide the effects of loss of a metabolic/glycolytic oscillator on oscillations in secretion. Therefore, secretion from perfused islets was examined in the presence of diazoxide (to keep K_{ATP} channels open) and high KCl (to depolarize the cell directly and keep [Ca^{2+}], high). Oscillations in insulin secretion were still seen from PFK-M-deficient islets (as well as from wild-type islets; Fig. 6) of similar period and amplitude as in the absence of diazoxide/KCl.

DISCUSSION

This mouse model with a disruptive tag inserted into the PFK-M gene is particularly interesting because of the tissue-dependent deficiency in PFK-M, with muscle and heart being less or little affected compared with 99% loss in brain and 95–98% loss in pancreatic islets. The results provide further evidence for the two-promoter system of the PFK-M gene, with its alternative initiation sites of the PFK-M message. The mouse and human PFK-M genes contain two promoters: a distal promoter that is expressed in all tissues and a proximal promoter that can be regulated by muscle-specific enhancers (16, 28–30, 52). By comparing a downstream sequence provided by Lexicon Genetics with the mouse PFK-M genomic sequence, we ascertained that the OmniBank gene trap vector was inserted at position −3,273 to −3,272 relative to the ATG start site. The proximal transcript 5′-untranslated region has been mapped to −106 to −9 bp, whereas the distal transcript 5′-untranslated region has been mapped to −3,612 to −3,550, with a putative splice junction between −3,550 and −3,549 (29). Both transcripts share the −8 to −1 sequence. Thus the disrupting tag is between the two promoters and close to the distal promoter. This disrupts the functioning of the distal
promoter, as shown by the RT-PCR data in Table 1, and therefore, tissues such as brain and presumably islet that rely on the distal promoter have greatly reduced PFK-M. Muscle and heart utilize the proximal promoter as well as the distal promoter. The 50–75% reduction in PFK-M protein in muscle in PFK-M-deficient mice may suggest that there is normally a substantial contribution of the distal promoter to PFK-M protein production in muscle or that the action of upstream sequences controlling the proximal promoter was also affected, as indicated by the 50% decrease in proximal transcript (Table 1). Nevertheless, because of the great surplus of PFK-M in muscle (45), this limited reduction may have little physiological consequence. This may be the reason that the PFK-M-deficient mice did not show insulin resistance in contrast to that reported in some studies of humans with global loss of PFK-M (41).

Heart showed little reduction in PFK-M protein in these mice, and the relative amount of the proximal transcript was increased. Perhaps there are differences in the muscle-specific enhancers or their potency in heart vs. skeletal muscle. Conversely, there could be loss of regulatory sequences that restrict transcription and are normally more potent in heart. In addition, on a translational level, Nakajima et al. (29) noted that the 5'-untranslated region in the proximal promoter transcript contained putative regulatory sequences that might affect the translation efficiency.

The two-promoter system has also been found in the rat PFK-M gene. However, in contrast to mouse and human, in rat the proximal promoter appears to be operative in nonmuscle tissues such that brain had as much proximal transcript as distal transcript (26). Recently, a third type of PFK-M transcript has been found in mouse and human that is initiated much further upstream from the distal promoter and is specific to testis and embryo (51).

Interestingly, the PFK-M deficiency led to a deficiency in insulin secretion. The mice showed impaired glucose tolerance but were not insulin resistant, at least not to a degree detectable by the insulin tolerance test. The glucose-stimulated insulin levels in vivo were perhaps only somewhat lower than in wild-type mice, but the plasma glucose levels were considerably higher. Normalization to basal insulin showed a significantly lower percentage increase in insulin in the PFK-M-

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**Table 2. Oscillatory insulin secretion from perifused islets**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Wild Type</th>
<th>PFK-M Deficient</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Average Secretion, ng/ml</td>
<td>Amplitude, ng/ml</td>
</tr>
<tr>
<td>A</td>
<td>6.3</td>
<td>2.1±0.5</td>
</tr>
<tr>
<td>B</td>
<td>4.0</td>
<td>1.9±0.7</td>
</tr>
<tr>
<td>C</td>
<td>2.0</td>
<td>0.81±0.27</td>
</tr>
<tr>
<td>D</td>
<td>1.7</td>
<td>0.87±0.22</td>
</tr>
</tbody>
</table>

Data are means ± SE. Average secretion, amplitude (average height of peaks above baseline), and period (average peak-to-peak time interval) were calculated by Cluster analysis (48) of the raw (unaveraged) data of the respective experiments shown in Fig. 4, omitting the large first-phase peak in C and D.
deficient mice. Furthermore, their isolated perifused islets showed diminished glucose-stimulated insulin secretion. A simple explanation for this could be reduced glycolytic flux; however, this is unlikely to be the case because of the presence of the other PFK isoforms (which were actually increased in amount in the PFK-M-deficient islets, as shown in Fig. 1D) and the considerable reserve of PFK activity compared with the metabolic flux. Most likely, glucokinase, the glucose sensor for glycolytic flux (25), remains rate limiting. Trus et al. (47) reported PFK activities in islet extracts that were 40 times those of glucokinase, and even that may have been a gross underestimate because of the reported instability of PFK in the cell extracts; a companion paper (5) showed in addition that glucose usage at 10 mM glucose was equivalent to about 50% of the assayed glucokinase activity but to 1% of the assayed PFK activity. Note that, in brain, a tissue with a similar PFK isomor mix as islet, total assayed PFK activity was decreased only 50% when PFK-M was decreased 99% in the deficient mice. That glucokinase remains the rate-limiting step in the PFK-M-deficient islets is further indicated by the great stimulation of secretion by increased glucose concentration and the fact that the impairment was not much greater at higher (16.7 mM) than lower (11 mM) stimulatory glucose (Fig. 4 and Table 1). The observation that Ca^{2+} responses were normal suggests that the deficiency is in amplifying or incretin effects. It has been suggested that an elevated ATP/ADP ratio may be involved in downstream effects of glucose as well as the triggering of Ca^{2+} influx (17). A normal glucose-stimulated rise in the ATP/ADP ratio was seen in PFK-M-deficient islets. Although it is possible that the rise was impaired in the cytosol, but not detected in our whole cell assay because of the ADP in the insulin secretion granules and perhaps mitochondria (12), nevertheless, clearly it was sufficient to give a normal [Ca^{2+}]_{i} response. Another possible reason for diminished insulin secretion could be deficiency in lipid molecules, which are important in secretion (8, 53) and which we have shown enhance the amplitude of oscillations in secretion from perifused islets (11). Although the PFK-M-deficient mice were not consistently of lower body weight, at least in the age range used here, it did appear that they were considerably less fatty, a topic that is being pursued elsewhere. Finally, it is possible that the decreased secretion could be due to loss of PFK-M protein, independent of its enzymatic activity, since PFK is known to interact with at least three proteins that influence exocytosis, namely tubulin, calmodulin, and caveolin (6, 22, 31, 39).

Insulin secretion in vivo and from perifused islets is oscillatory with a period of minutes (10, 38, 44). Loss or impairment of the oscillations is seen in patients with type 2 diabetes and in their near relatives (34, 37), and this may be contributory to the development of the disease because oscillations enhance the potency of insulin (24, 35). On the basis of the spontaneous oscillations of glycosaly and the ATP/ADP ratio caused by autacatalytic F16BP activation of PFK in skeletal muscle extracts, we proposed that the oscillations in [Ca^{2+}]_{i}, and insulin secretion in the pancreatic β-cell are due to similar oscillations in glycosaly generated by PFK-M (9, 44). Oscillations in insulin secretion were still observed in PFK-M-deficient islets but were of reduced amplitude and diminished regularity. [Ca^{2+}]_{i} oscillations in clusters of cells appeared normal, and there was no great decrease in the number of oscillators even among isolated cells, arguing against the presence of a small number of unimpaired cells serving as pacemakers. Hence, superficially, one might conclude that PFK-M is not the oscillator, and that may indeed be the case. However, these islets had a residual 2–5% PFK-M, which may still have been sufficient to generate metabolic oscillations. As noted above, there is a large reserve of PFK activity compared with the metabolic flux. Metabolic oscillations have been observed in dilute extracts of muscle (1, 46) and islet (7). Thus it is not clear whether the [Ca^{2+}]_{i} oscillations and residual insulin oscillations in this mouse model are due to residual activity of PFK-M or to other PFK isoforms present in islets or may involve another oscillator. For example, a mitochondrial oscillator (23) of unknown mechanism is another possibility. On the other hand, in studies of a human family with inherited PFK-M deficiency, normal regular insulin oscillations (under basal conditions) were lost in the brother with a homozygous PFK-M deficiency (40). The human situation may be more complicated since the point mutations involved may lead to truncated, enzymatically inactive proteins that might affect heterotetramers, not just simple loss of PFK-M expression as in this mouse model. The decreased amplitude of insulin oscillations in the PFK-M-deficient mouse islets may reflect the overall decrease in secretion, that is, diminished incretin effects as noted above. The decreased regularity may reflect a less efficient synchronization of the group of islets perifused in the column.

Some other candidate models for generation of oscillations in the pancreatic β-cell involve feedback effects of [Ca^{2+}], (42). However, insulin oscillations were still observed here in the presence of diazoxide and high KCl, as we reported previously in rat islets (10). Others have also shown insulin oscillations from ob/ob mouse islets when [Ca^{2+}]_{i}, was constant (49). Our results show that this response is not restricted to islets from the hyperglycemic ob/ob mice, as suggested by some (21). This indicates that there is an oscillating mechanism that does not require oscillations in and, hence, feedback regulation by Ca^{2+} directly or indirectly on the K_{ATP} channel or on other channels or enzymes. The most likely possibility at this point is the existence of (at least) a glycolytic oscillator and a calcium/ionic oscillator, which can operate separately or interact with each other; mathematical modeling based on these concepts can account for the variety and complexity of oscillatory behavior observed in islets/β-cells (3, 4, 33). Under conditions when [Ca^{2+}]_{i} is oscillating (whether independently or driven by a metabolic oscillator), it may be the major factor causing the oscillatory changes in insulin secretion. However, clearly other, presumably metabolic factors can also contribute, as shown here and elsewhere (19). In the PFK-M-deficient islets, the [Ca^{2+}]_{i} oscillations and residual insulin oscillations could possibly be due to the calcium/ionic oscillator, but the experiments with diazoxide and high KCl indicate that a metabolic oscillator is still operative, too.

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