Glucose Metabolism and Pulsatile Insulin Release From Isolated Islets

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The effects of metabolic inhibition on insulin release and the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) were studied in individually perifused pancreatic islets from *ob/ob* mice. The modest basal secretion in the presence of 3 mmol/l glucose was pulsatile with a frequency of ~0.2/min, although $[Ca^{2+}]_i$ was stable at ~100 nmol/l. Introduction of 11 mmol/l glucose resulted in large amplitude oscillations of $[Ca^{2+}]_i$ and almost 20-fold stimulation of average secretion manifested as increased amplitude of the insulin pulses without change in frequency. Inhibition of glycolysis with iodoacetamide or mitochondrial metabolism with dinitrophenol or antimycin A reduced glucose-stimulated secretion back to basal levels with maintained pulsatility. The $[Ca^{2+}]_i$ responses to the metabolic inhibitors were more complex, but in general there was an initial peak and eventually sustained elevation without oscillations. When introduced in the presence of 3 mmol/l glucose, the metabolic inhibitors tended to increase the amplitude of the insulin pulses, although the simultaneous elevation in $[Ca^{2+}]_i$ occurred without oscillations. The data indicate that pulsatile secretion is regulated by factors other than $[Ca^{2+}]_i$ under basal conditions and after metabolic inhibition. Although pulsatile secretion can be driven by oscillations in metabolism when $[Ca^{2+}]_i$ is stable, it was not possible from the present data to determine whether insulin pulses have a glycolytic or mitochondrial origin. *Diabetes* 50:1785–1790, 2001

The circulating insulin concentration displays regular oscillations in normal human subjects (1). Disturbances of these oscillations are an early sign in the development of type 2 diabetes (2,3). Such alterations may lead to insulin resistance and glucose intolerance by downregulation of insulin receptors (4). The plasma insulin oscillations have been attributed to the pulsatile release of the hormone from the pancreas (5), a process that requires coordinated secretion from the pancreatic islets (6).

When the ambient glucose concentration is raised, β-cell metabolism is stimulated (7). The resulting increase in the ATP/ADP ratio decreases the permeability of the ATP-sensitive K^+ (K_{ATP}) channels and depolarizes the cell (8). Subsequent influx of Ca^{2+} through voltage-dependent channels triggers the exocytosis of insulin granules (9). ATP produced by metabolism also plays an important role in providing energy for secretion by recruitment and priming of insulin granules for exocytosis (10). Impaired glucose metabolism in pancreatic β-cells has been implicated in the development of the disturbed plasma insulin pattern seen in type 2 diabetes (11). Support for this idea has been obtained from altered plasma insulin patterns in patients with mutations in genes coding for glycolytic enzymes, such as phosphofructokinase and glucokinase and mitochondrial tRNA (12–17).

In the present study, we attempted to determine the importance of glycolytic and mitochondrial metabolism for pulsatile insulin release by measuring insulin secretion and $[Ca^{2+}]_i$ from individual pancreatic islets exposed to metabolic inhibitors at basal and stimulatory glucose concentrations.

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$[Ca^{2+}]_i$, cytoplasmic Ca^{2+} concentration; DNP, 2,4-dinitrophenol; IAA, iodoacetamide; K_{ATP} channel, ATP-sensitive K^+ channel.

RESEARCH DESIGN AND METHODS

Materials. Reagents of analytical grade and deionized water were used. Collagenase, HEPES, and bovine serum albumin (fraction V) were obtained from Boehringer Mannheim (Mannheim, Germany). Antimycin A, iodoacetamide (IAA), 2,4-dinitrophenol (DNP), tetramethylbenzidine, and insulin-peroxidase were obtained from Sigma (St. Louis, MO). The rat insulin standard was obtained from Novo Nordisk (Bagsvaerd, Denmark). IgG-certified microtiter plates were purchased from Nunc (Roskilde, Denmark). The insulin antibodies were raised in guinea pigs.

Preparation and culture of islets. Pancreatic islets were collagenase-isolated from *ob/ob* mice taken from a local colony (18). For the insulin experiments, freshly isolated islets were perifused in a medium supplemented with 1 mg/ml albumin and containing (in mmol/l) 125 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 1.3 $CaCl_2$, and 25 HEPES, titrated to pH 7.4 with NaOH. For the $[Ca^{2+}]_i$ experiments, the islets were cultured overnight in the presence of 5.5 mmol/l glucose in RPMI 1640 supplemented with 10% fetal calf serum, and the Ca^{2+} concentration of the perifusion medium was 2.6 mmol/l.

Measurements of insulin release. The kinetics of insulin release were studied essentially as described previously (6). A single islet was placed in a temperature-controlled (37°C) 10-μl chamber and perifused with medium at 150 μl/min with the aid of a peristaltic pump placed before the chamber. After 60–75 min of introductory perifusion in the presence of 3 mmol/l glucose, the perifusate was collected in 20-s fractions directly into microtiter plates. Insulin was assayed by a competitive enzyme-linked immunosorbent assay with the insulin antibody immobilized directly onto the solid phase. Amounts of insulin down to 100 amol were obtained from linear standard curves in semilogarithmic plots. The rate of insulin release was normalized to dry weight after the islets were freeze-dried and weighed on a quartz fiber balance. For establishing the precision of the assay under the present experimental conditions, empty chambers were perfused with known concentrations of insulin. These studies clarified that the experimental procedures per se do not give rise to oscillatory variations.

Measurements of $[Ca^{2+}]_i$. Individual islets were loaded with 2 μmol/l fura-2 acetoxymethyl ester in the presence of 3 mmol/l glucose for 50 min. Each islet was attached to a poly-L-lysine–coated coverslip serving as the bottom of a 150-μl perifusion chamber. The chamber was placed on the stage of an inverted microscope (Nikon Diaphot, Nikon, Tokyo) within a climate box maintained at 37°C. $[Ca^{2+}]_i$ was recorded using dual wavelength fluorometry

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with excitation at 340 and 380 nm and emission at 510 nm as described previously (19,20).

Statistical analysis. The frequency of pulsatile insulin release and $[Ca^{2+}]_i$ was determined by Fourier transformation using the Igor software (Wave Metrics, Lake Oswego, OR). Results are presented as means ± SE. Differences in secretory rates were evaluated with one-way analysis of variance (ANOVA).

RESULTS

Basal insulin release from individual islets that were exposed to 3 mmol/l glucose exhibited pronounced pulses with a frequency of 0.23 ± 0.01/min (n = 11), although the average rate of secretion was modest, corresponding to 6.9 ± 0.7 $pmol \cdot g^{-1} \cdot s^{-1}$ (Figs. 1 and 2, Tables 1–3). When the glucose concentration was raised to 11 mmol/l, there was an almost 20-fold increase in average insulin secretion, reaching 118 ± 37 $pmol \cdot g^{-1} \cdot s^{-1}$. This was due to an increase in the amplitude of the insulin pulses without change in frequency (0.19 ± 0.01/min; Fig. 1, Tables 1–3). Introduction of 1 mmol/l of the glycolytic inhibitor IAA in the presence of 11 mmol/l glucose inhibited secretion back to basal levels but did not prevent continued pulsatility with similar frequency (Fig. 1*A* and Table 1). The mitochondrial inhibitors DNP (250 μmol/l) and antimycin A (10 μmol/l) mimicked the effect of IAA, reducing secretion to basal levels with maintained pulsatility (Figs. 1*B* and *C*, Tables 2 and 3).

The basal $[Ca^{2+}]_i$ levels in the presence of 3 mmol/l glucose were stable at ~100 nmol/l. An increase of glucose to 11 mmol/l resulted in initial lowering of $[Ca^{2+}]_i$ followed by a pronounced peak and subsequent oscillations (Fig. 3). The major frequency component of these oscillations was 0.42 ± 0.08/min. In the presence of the latter glucose concentration, introduction of IAA caused sustained elevation of $[Ca^{2+}]_i$ followed by a decrease that approached basal levels in 10 min and then a gradual slow increase (Fig. 3*A*). The mitochondrial inhibitors DNP and antimycin A had more pronounced effects, inducing a prominent transient elevation of $[Ca^{2+}]_i$ followed by a sustained elevated level (Figs. 3*B* and *C*).

When metabolic inhibitors were introduced in the presence of the basal glucose concentration (3 mmol/l), the effect on insulin release tended to be slightly stimulatory with a gradual increase in the amplitude of the insulin pulses, although significance was not reached within 20 min (Fig. 2 and Tables 1–3). Also, in this case, the frequency of the insulin pulses remained unaffected. In parallel experiments $[Ca^{2+}]_i$ was found to be low and stable in the presence of 3 mmol/l glucose. The introduction of IAA caused a slow, gradual increase in $[Ca^{2+}]_i$ (Fig. 4*A*), whereas mitochondrial inhibition with DNP (Fig. 4*B*) or antimycin A (Fig. 4*C*) induced an initial prompt increase followed by sustained elevation, similar to the response at the stimulatory glucose concentration.

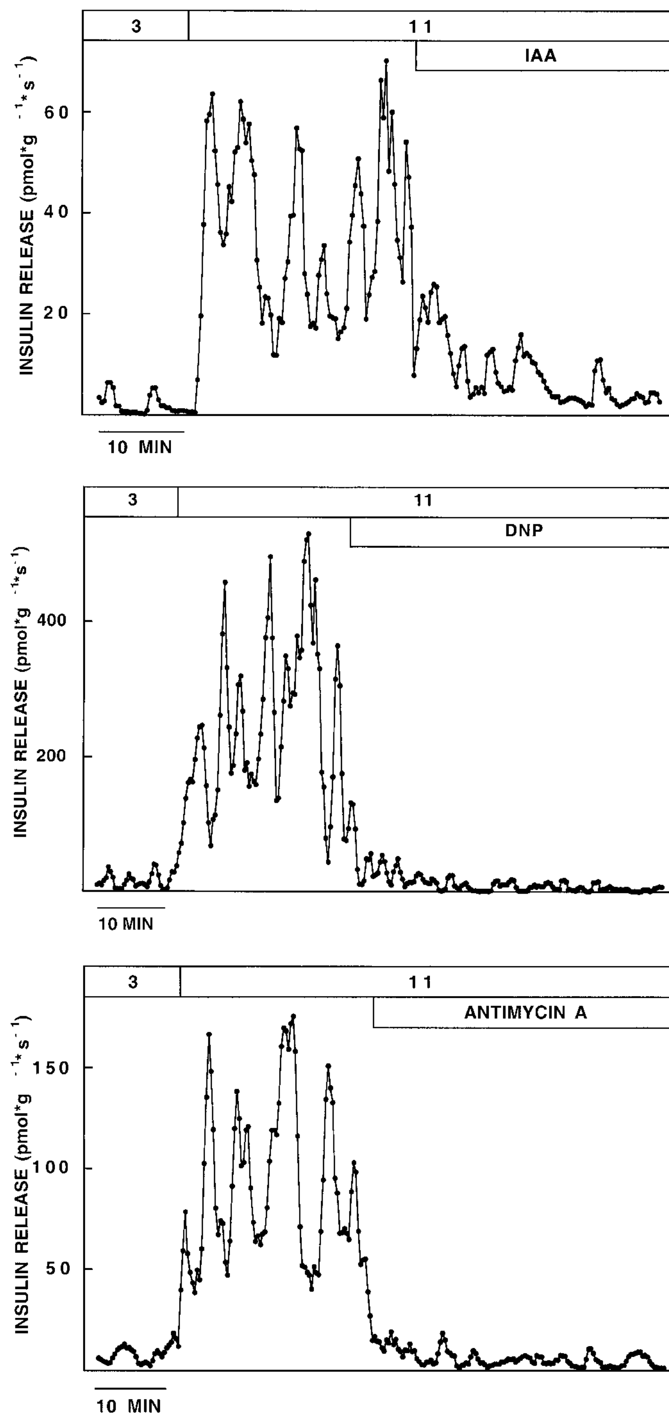

FIG. 1. Effects of metabolic inhibitors on glucose-induced insulin release. Individual islets were perifused in the presence of 3 and 11 mmol/l glucose, as indicated by the bars. Lower bars show addition of 1 mmol/l IAA (*A*), 250 μmol/l DNP (*B*), or 10 μmol/l antimycin A (*C*). Results are representative of three (*A* and *B*) or five (*C*) experiments.

DISCUSSION

The pancreatic β-cells are central for glucose homeostasis by sensing the blood glucose concentration and releasing insulin as required. Given that this is an energy-dependent process (21), it is not surprising that metabolic inhibitors dramatically reduce insulin release (22–27). However, such inhibitors have more profound effects on the β-cell, because the cell's recognition of glucose as a secretory stimulus also depends on metabolism of the glucose (28). An increase in the ATP/ADP ratio seems to be the crucial factor, both supplying energy for secretion (10) and coupling metabolism to depolarization by inhibiting K^+ efflux through the K_{ATP} channels (29). Subsequent opening of voltage-dependent Ca^{2+} channels results in influx of the ion and rise of $[Ca^{2+}]_i$ (30), the most important trigger of the exocytosis of the insulin granules (31).

During glucose stimulation, there are marked oscillations of $[Ca^{2+}]_i$ (32), which are paralleled by pulsatile

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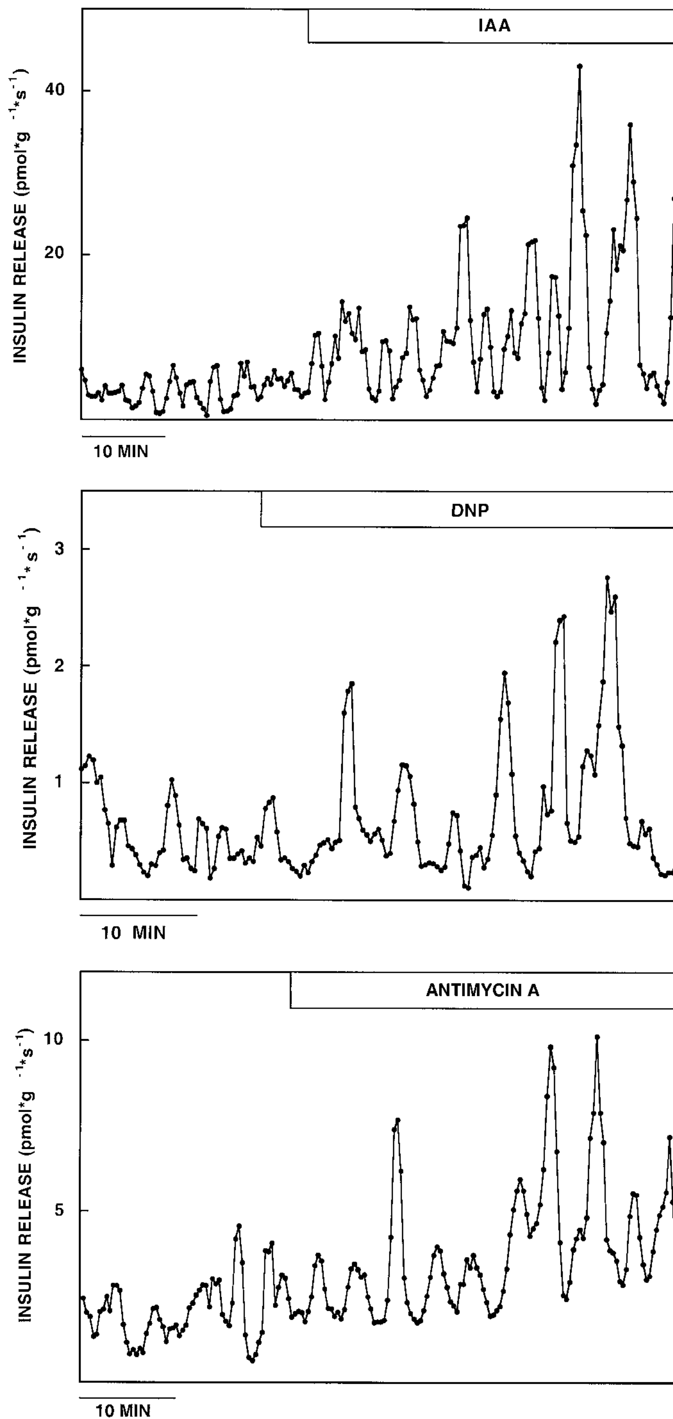

FIG. 2. Effects of metabolic inhibitors on basal insulin release. Individual islets were perifused in the presence of 3 mmol/l glucose. Bars show addition of 1 mmol/l IAA (*A*), 250 μmol/l DNP (*B*), or 10 μmol/l antimycin A (*C*). Results are representative of three (*A* and *B*) and five (*C*) experiments.

insulin release (19,33,34). Also, the ATP/ADP ratio is believed to oscillate, but there are different ideas about the mechanisms involved. According to one opinion, each $[Ca^{2+}]_i$ peak reduces the ratio by stimulating the energy-requiring removal of the ion from the cytoplasm, but metabolism is not oscillating per se (35). Alternatively, there are primary oscillations in metabolism driving those of $[Ca^{2+}]_i$ (36,37). In support of the latter view, there are oscillations in K_{ATP} channel activity under conditions when $[Ca^{2+}]_i$ is low and stable (38). Moreover, the oxygen consumption of pancreatic islets exhibits oscillations when $[Ca^{2+}]_i$ is kept at resting levels by blocking the voltage-dependent entry of the ion (39).

If primary oscillations in metabolism underlie those in the ATP/ADP ratio and $[Ca^{2+}]_i$, then it can be anticipated that pulsatile secretion is driven by two parallel signals, the former providing energy and the latter acting as trigger. It is pertinent to note, therefore, that also the faint basal release of the hormone is pulsatile when $[Ca^{2+}]_i$ is low and stable (40). Indeed, even stimulated secretion maintains its pulsatility when experimentally clamping $[Ca^{2+}]_i$ at stably elevated levels (41,42). Consequently, it seems as though oscillations in either the ATP/ADP ratio or $[Ca^{2+}]_i$ can support pulsatile secretion when the other factor remains stable (41,43). What, then, is the origin of oscillations in metabolism? A glycolytic oscillator has been proposed on the basis of the observation that the β-cell expresses the same isoform of phosphofructokinase, which is a prerequisite for glycolytic oscillations in muscle extracts (44,45). However, this view is challenged by the observations that mitochondrial substrates such as leucine and α-ketoisocaproic acid induce oscillations of $[Ca^{2+}]_i$ (46,47) and pulsatile insulin release (47,48).

In accordance with preceding studies, we now found that basal insulin secretion is pulsatile when $[Ca^{2+}]_i$ is low and stable (40) and that pulsatile secretion in response to glucose occurs when there are oscillations in $[Ca^{2+}]_i$ (6). Because the present measurements of $[Ca^{2+}]_i$ and secretion were not performed in parallel, we could not confirm the previously demonstrated synchronization (19,33). However, the major frequency component in the $[Ca^{2+}]_i$ measurements was within the range expected from studies of $[Ca^{2+}]_i$ and insulin release (19,33,34). Upon introduction of meta-

TABLE 1
Effects of IAA on basal and stimulated insulin release

Glucose (mmol/l)	IAA (mmol/l)	Insulin release		n
		$pmol \cdot g^{-1} \cdot s^{-1}$	osc/min	
3	—	5.1 ± 1.9	0.23 ± 0.04	6
3	1	7.6 ± 3.1	0.17 ± 0.03	3
11	—	97 ± 17*	0.21 ± 0.03	3
11	1	8.3 ± 1.2†	0.22 ± 0.04	3

Individual islets were perifused in the presence of 3 mmol/l glucose. IAA (1 mmol/l) was added to the perifusion medium either directly or after first elevating the glucose concentration to 11 mmol/l. *$P < 0.001$ vs. 3 mmol/l glucose alone; †$P < 0.001$ vs. 11 mmol/l glucose alone. osc, oscillations.

TABLE 2
Effects of DNP on basal and stimulated insulin release

Glucose (mmol/l)	DNP (μmol/l)	Insulin release		n
		$pmol \cdot g^{-1} \cdot s^{-1}$	osc/min	
3	—	7.2 ± 0.8	0.22 ± 0.02	6
3	250	9.6 ± 0.8	0.19 ± 0.03	3
11	—	108 ± 25*	0.19 ± 0.02	3
11	250	8.8 ± 2.6†	0.19 ± 0.04	3

Individual islets were perifused in the presence of 3 mmol/l glucose. DNP (250 μmol/l) was added to the perifusion medium either directly or after first elevating the glucose concentration to 11 mmol/l. *$P < 0.001$ vs. 3 mmol/l glucose alone; †$P < 0.001$ vs. 11 mmol/l glucose alone. osc, oscillations.

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TABLE 3
Effects of antimycin A on basal and stimulated insulin release

Glucose (mmol/l)	Antimycin A (μmol/l)	Insulin release pmol · g^{-1} · s^{-1}	Insulin release osc/min	n
3	—	6.4 + 0.6	0.21 + 0.02	10
3	10	10.4 + 2.7	0.18 + 0.03	5
11	—	139 + 24*	0.19 + 0.03	5
11	10	5.9 + 1.5†	0.17 + 0.03	5

Individual islets were perifused in the presence of 3 mmol/l glucose. Antimycin A (10 μmol/l) was added to the perifusion medium either directly or after first elevating the glucose concentration to 11 mmol/l. *$P < 0.001$ vs. 3 mmol/l glucose alone; †$P < 0.001$ vs. 11 mmol/l glucose alone. osc, oscillations.

bolic inhibitors, the glucose-stimulated secretion was reduced back to basal levels and the release process became dissociated from $[Ca^{2+}]_i$. The early elevation of $[Ca^{2+}]_i$ induced by the inhibitors consequently has no counterpart in the secretory pattern. Moreover, the pulsatile pattern of secretion was maintained when $[Ca^{2+}]_i$ did not display oscillations in the presence of the inhibitors, as was shown previously under other conditions (34,40–42).

DNP is an uncoupler that dissipates the H^+ gradient, which drives the ATP-synthase in the inner mitochondrial membrane, and antimycin A blocks the electron transport chain at site II. The maintained pulsatility of secretion in the presence of these mitochondrial inhibitors may be taken to indicate that a putative glycolytic oscillator can operate without disturbance. However, the finding that pulsatility persists in the presence of IAA, which exerts its major effect by alkylating the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, is instead an argument for a mitochondrial oscillator. Thus, it was difficult from the present data to determine whether insulin pulses have a glycolytic or a mitochondrial origin. In the absence of glycolysis, energy essentially may be derived from mitochondrial oxidation of endogenous fat. The metabolism of endogenous substrates in pancreatic islets that are exposed to nutrient-free medium is substantial, being almost half of that observed during maximal glucose stimulation (21,49). The more pronounced effects of the mitochondrial than glycolytic inhibitors on $[Ca^{2+}]_i$ in the presence of 3 mmol/l glucose can be taken as an argument for a greater contribution of mitochondrial substrates to the basal metabolism.

Basal insulin secretion is stimulated slightly by metabolic inhibition (25). In the present study, this effect became apparent as a small increase in the amplitude of the insulin pulses. Although significance was not reached for any inhibitor, the tendency was similar irrespective of whether mitochondrial or glycolytic metabolism was compromised. These similarities are somewhat surprising because the mitochondrial inhibitors had more dramatic effects on $[Ca^{2+}]_i$, inducing an initial peak followed by sustained increase rather than the slight gradual elevation obtained with IAA. It seems that a rise of $[Ca^{2+}]_i$ is a weak stimulus for secretion when the ATP/ADP ratio is low. It remains to be established whether the more dramatic effects of the mitochondrial inhibitors on $[Ca^{2+}]_i$ reflect a direct interference with the Ca^{2+} handling of this organelle, possibly by decreasing its membrane potential, or simply are more effective in reducing the ATP/ADP ratio.

Even under normal conditions, as much as 20% of the cellular ATP is required for maintenance of ion gradients (21). In this context, it is important to point out that whereas the Ca^{2+} signal is derived primarily from superficially located fura-2–loaded islet cells, insulin release measurements originate from both superficially and centrally located β-cells in the islet. The possibility that superficial cells may be more affected by the inhibitors than more centrally located cells may help to explain the

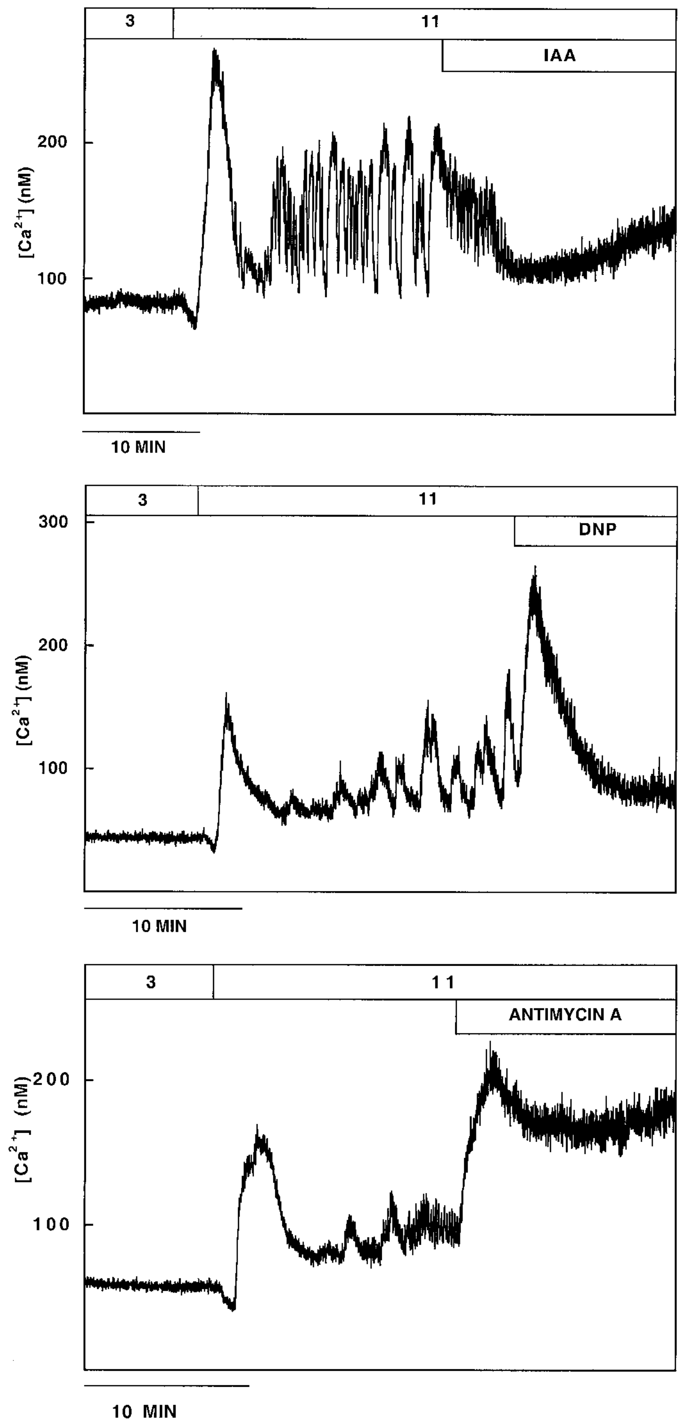

FIG. 3. Effects of metabolic inhibitors on $[Ca^{2+}]_i$ of glucose-stimulated pancreatic islets. Individual murine islets were perifused in the presence of 3 and 11 mmol/l glucose, as indicated by the bars. Lower bars show addition of 1 mmol/l IAA (*A*), 250 μmol/l DNP (*B*), or 10 μmol/l antimycin A (*C*). Results are representative of three experiments.

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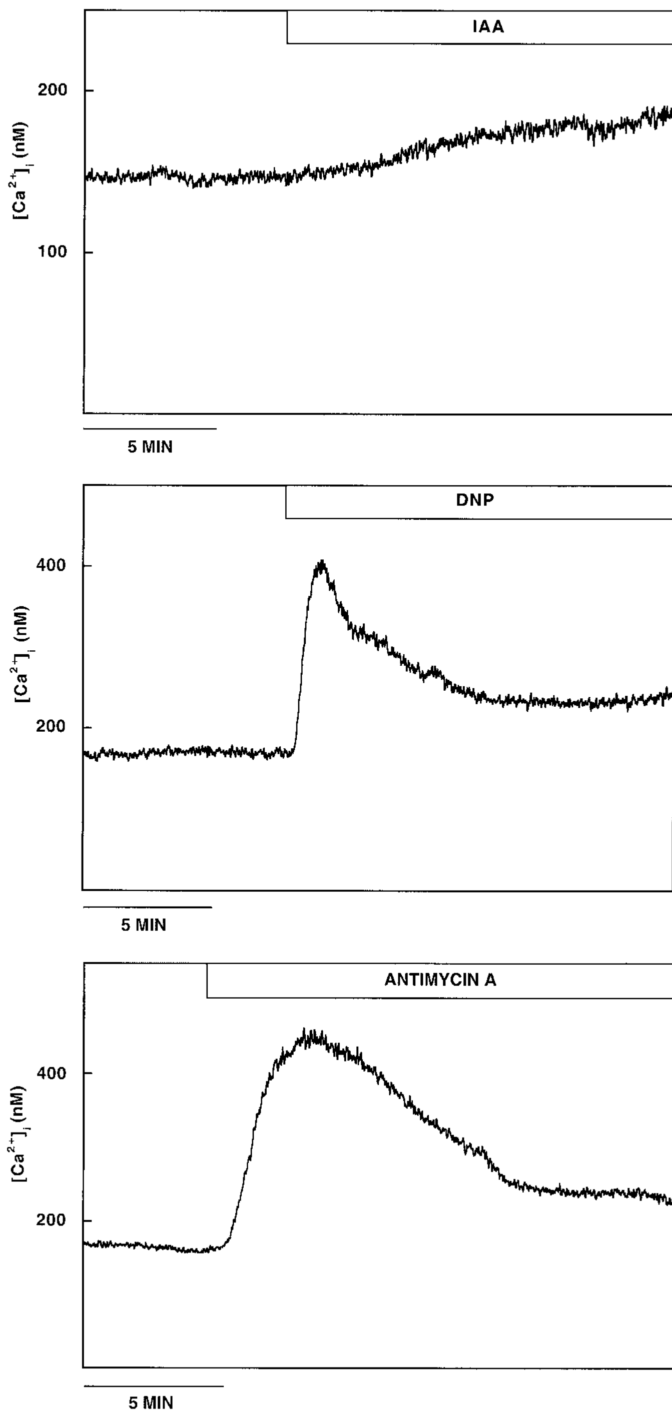

FIG. 4. Effects of metabolic inhibitors on $[Ca^{2+}]_i$ of islets that were exposed to basal glucose concentration. Individual islets were perifused in the presence of 3 mmol/l glucose. Bars show addition of 1 mmol/l IAA (*A*), 250 μmol/l DNP (*B*), or 10 μmol/l antimycin A (*C*). Results are representative of two (*A* and *B*) and three (*C*) experiments.

differences between the $[Ca^{2+}]_i$ and insulin release measurements in the presence of the inhibitors.

Although the metabolic inhibitors reduce insulin secretion to basal levels, the pulsatility is maintained, indicating that the release process is still regulated. It is puzzling that the frequency of the pulses is identical irrespective of glycolytic or mitochondrial inhibition. However, it seems unlikely that there are separate glycolytic and mitochondrial oscillators with similar frequency. Other studies have shown that inhibitors, identical or similar to those currently used, fail to abolish metabolism completely (27,50), perhaps providing an explanation for the maintained pulsatility. Future studies that will examine measurements of the ATP/ADP ratio and the effects of fatty acids on insulin release will be important to gain more insight into the origin of the insulin pulses.

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