Oscillations of Insulin Secretion Can Be Triggered by Imposed Oscillations of Cytoplasmic Ca^{2+} or Metabolism in Normal Mouse Islets

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Glucose-induced insulin secretion depends on an acceleration of glucose metabolism, requires a rise in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$), and is modulated by activation of protein kinases in β-cells. Normal mouse islets were used to determine whether oscillations of these three signals are able and necessary to trigger oscillations of insulin secretion. The approach was to minimize or abolish spontaneous oscillations and to compare the impact of forced oscillations of each signal on insulin secretion. In a control medium, repetitive increases in the glucose concentration triggered oscillations in metabolism [NAD(P)H fluorescence], $[Ca^{2+}]_i$ (fura-PE3 method), and insulin secretion. In the presence of diazoxide, metabolic oscillations persisted, but $[Ca^{2+}]_i$ and insulin oscillations were abolished. When the islets were depolarized with high K^+ with or without diazoxide, $[Ca^{2+}]_i$ was elevated, and insulin secretion was stimulated. Forced metabolic oscillations transiently decreased or did not affect $[Ca^{2+}]_i$ and potentiated insulin secretion with oscillations of small amplitude. These oscillations of secretion followed metabolic oscillations only when $[Ca^{2+}]_i$ did not change. When $[Ca^{2+}]_i$ fluctuated, these changes prevailed over those of metabolism for timing secretion. Repetitive depolarizations with high K^+ in the presence of stable glucose (10 mmol/l) induced synchronous pulses of $[Ca^{2+}]_i$ and insulin secretion with only small oscillations of metabolism. Continuous stimulation of protein kinase A (PKA) and protein kinase C (PKC) did not dissociate the $[Ca^{2+}]_i$ and insulin pulses from the high K^+ pulses. However, the amplitude of the insulin pulses was consistently increased, whereas that of the $[Ca^{2+}]_i$ pulses was either increased (PKA) or decreased (PKC). In conclusion, metabolic oscillations can induce oscillations of insulin secretion independently of but with a lesser effectiveness than $[Ca^{2+}]_i$ oscillations. Although oscillations in metabolism may cyclically influence secretion through an ATP-sensitive K^+ channel (K^+-ATP channel)-independent pathway, their regulatory effects are characterized by a hysteresis that makes them unlikely drivers of fast oscillations, unless they also involve $[Ca^{2+}]_i$ changes through the K^+-ATP channel–dependent pathway. *Diabetes* 48:2374–2382, 1999

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Received for publication 29 March 1999 and accepted in revised form 24 August 1999.

$[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; K^+-ATP channel, ATP-sensitive K^+ channel; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

A rise in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in β-cells is essential for glucose stimulation of insulin secretion. This rise is the outcome of a now well-established sequence of events (1–5). Glucose metabolism generates signals, including an increase in the ATP:ADP ratio (6), that close ATP-sensitive K^+ channels (K^+-ATP channels) in the plasma membrane. The resulting membrane depolarization opens voltage-dependent Ca^{2+} channels, thereby promoting Ca^{2+} influx and a rise in $[Ca^{2+}]_i$. Insulin secretion can also be potentiated by an increase in the efficacy of Ca^{2+} on exocytosis. This is achieved either by glucose itself through a mechanism known as the K^+-ATP channel–independent pathway (7–11) or by hormones and neurotransmitters that ultimately activate protein kinases (1,12–14).

Insulin secretion is a pulsatile process, and this pulsatility is important for normal glucose homeostasis (15–17). Several in vitro studies have established that oscillations of insulin secretion are temporally correlated with $[Ca^{2+}]_i$ oscillations in β-cells, which suggests that Ca^{2+} is a major regulator of pulsatility (18–21). Dissociations between both phenomena have, however, been reported and have led to the proposal that Ca^{2+} has a permissive rather than a regulatory role (22–24). A metabolic or biochemical signal may change cyclically and be the true regulator of the oscillations of insulin secretion (25).

The purpose of the present study was not to identify and characterize the oscillatory events occurring spontaneously in glucose-stimulated β-cells nor to investigate their possible mechanisms. These topics are complex and are subjects of controversy. Our aim was to determine whether oscillations of $[Ca^{2+}]_i$, metabolism, and protein kinase activity are able and necessary to induce oscillations of insulin secretion. Under physiological conditions, the three factors may well oscillate together. Ideally, one should investigate each type of oscillator independently from the others during continuous stimulation with glucose in a control medium. Practically, this is not possible because of the interplay between these oscillators. For example, the reciprocal links between metabolism and $[Ca^{2+}]_i$ are such that an interference with spontaneous oscillations of the former will have repercussions on the oscillations of the latter (25–28). Evaluation of the respective role of each oscillator, therefore, requires control of one or the other two, which imposes the use of nonphysiological experimental conditions. Combined oscillations of metabolism and $[Ca^{2+}]_i$ were induced by alternating between low and high glucose concentrations in a control medium with

4.8 mmol/l K^+. Isolated metabolic oscillations were triggered by repetitive glucose applications in the presence of diazoxide, which, by opening K^+-ATP channels, prevents glucose from depolarizing the membrane and increasing $[Ca^{2+}]_i$ (7,9). Metabolic oscillations were also induced when $[Ca^{2+}]_i$ was maintained at an elevated level by depolarizing the β-cell membrane by 30 mmol/l K^+ with or without diazoxide. Large $[Ca^{2+}]_i$ oscillations with only small metabolic oscillations were induced by repetitively changing the extracellular K^+ concentration from 4.8 to 30 mmol/l in the presence of constant glucose with or without diazoxide. Finally, the activity of protein kinase A (PKA) and protein kinase C (PKC) was steadily increased by pharmacological agents.

RESEARCH DESIGN AND METHODS

Preparation and solutions. Islets were aseptically isolated by collagenase digestion of the pancreas of fed female NMRI mice (25–30 g) followed by hand selection (29). The islets were then cultured for 1 day in RPMI-1640 medium (Gibco, Paisley, U.K.) containing 10 mmol/l glucose, 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin. The control medium used for islet isolation was a bicarbonate-buffered solution containing (in mmol/l): NaCl 120, KCl 4.8, $CaCl_2$ 2.5, $MgCl_2$ 1.2, and $NaHCO_3$ 24. It was gassed with O_2/CO_2 (94/6) to maintain a pH of 7.4 and contained 10 mmol/l glucose and 1 mg/ml bovine serum albumin. The same medium was used for some experiments, but for many others, the concentration of KCl was increased to 30 mmol/l, whereas that of NaCl was decreased accordingly to maintain iso-osmolarity.

Measurement of $[Ca^{2+}]_i$. Cultured islets were loaded with fura-PE3 during 90 min of incubation at 37°C in the presence of 2 µmol/l fura-PE3 acetoxymethylester (Mobitec, Göttingen, Germany). A control medium with 10 mmol/l glucose was used during the first 60 min and was replaced by the same medium as that used at the start of the experiment for the last 30 min. Islets loaded with the dye were then transferred into a perifusion chamber (Intracell; Royston, Herts, U.K.) with a bottom made of a coverslip that was mounted on the stage of an inverted microscope. The islets were held in place by gentle suction with glass micropipettes. The preparation was perifused at a flow rate of 2 ml/min. The dead space of the system (1 min) has been corrected for in the figures. The solutions were kept at 38°C in a water bath, and a temperature controller ensured a temperature of 37 ± 0.3°C (range) in the chamber as monitored by a thermistor. The measurements of $[Ca^{2+}]_i$ were performed with the MagiCal system (Applied Imaging, Sunderland, U.K.) as described in detail (30). The tissue was excited at 340 and 380 nm during two successive periods of 0.32 s separated by an interval of 0.6 s. The illumination was then stopped for 2.25 s. The fluorescence emitted at 510 nm was captured by a charge-coupled device (CCD) videocamera (Photonic Science, Turnbridge Wells, U.K.). Successive images (ratios) were thus obtained at 3.5-s intervals for a total duration of 42 min (set by the technical characteristics of the system). From the ratio of the fluorescence at 340 nm and 380 nm, the concentration of $[Ca^{2+}]_i$ at each pixel was calculated by comparing it with a calibration curve (30). The mean $[Ca^{2+}]_i$ in the islet was then calculated by averaging the $[Ca^{2+}]_i$ at all pixels of the islet. Each experiment was usually performed with three islets from the same preparation (culture) and was repeated with islets from four to eight distinct preparations. Results are shown as means ± SE.

Measurement of reduced pyridine nucleotide fluorescence. Cultured islets were first preincubated for 60 min at 37°C in a control medium containing 10 mmol/l glucose and then for 30 min in the same medium as that used at the start of the experiment. The islets were then transferred to the same experimental setup that was used for the $[Ca^{2+}]_i$ measurements. The reduced forms of NAD and NADP [referred to as "NAD(P)H"] were excited at 360 nm for 0.64 s, and the fluorescence emitted was filtered at 470 nm (30). The illumination was then stopped for 1.15 s. Successive images were thus obtained at 1.8-s intervals for the same total duration as in $[Ca^{2+}]_i$ measurements. The mean NAD(P)H fluorescence in the islet was then calculated by averaging the signal recorded at all pixels of the islet. The changes in fluorescence were expressed as a percentage of basal values by dividing the integrated gray levels at a given time by those obtained during the last 40 s preceding the first application of a test agent. Each experiment was usually performed with three islets from the same preparation and was repeated with islets from four to eight distinct preparations. Results are shown as means ± SE.

Measurement of insulin secretion. Cultured islets were preincubated for 60 min at 37°C in a control medium containing 10 mmol/l glucose. Batches of 12–18 islets (depending on the experimental conditions) were then placed in perifusion chambers (31) and were perifused at a flow rate of 1.5 ml/min. The dead space of the system (1 min) has been corrected for in the figures. After 30 min of perifusion, effluent fractions were collected at 30-s intervals during the first 30 min and then at 60-s intervals. The experiments lasted longer than for the $[Ca^{2+}]_i$ and NAD(P)H measurements (74 vs. 42 min), but the same rhythm of stimulations was imposed. Insulin was measured with a double-antibody radioimmunoassay with rat insulin as the standard (Novo Research Institute, Bagsvaerd, Denmark). Each protocol was repeated with islets from four to nine distinct preparations. Results are presented as means ± SE.

Combined measurements of insulin secretion and $[Ca^{2+}]_i$ or pyridine nucleotides. This technique has been described in detail (29). In brief, one single islet was used at a time for measurement of $[Ca^{2+}]_i$ or the fluorescence of pyridine nucleotides as described above. The chamber (110 µl) was perfused at a flow rate of 1.8 ml/min, and the medium was collected in fractions of 30 s just downstream from the islet. Insulin was measured in duplicate 400-µl aliquots of the effluent fractions. The characteristics of the assay have previously been reported (29). The same protocol was repeated four times for $[Ca^{2+}]_i$ and NAD(P)H measurements; hence, insulin secretion was measured eight times with islets from four distinct preparations. Results are presented as means ± SE.

Reagents. Diazoxide was obtained from Schering-Plough Avondale (Rathdrum, Ireland), forskolin was obtained from Calbiochem (San Diego, CA), and phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma Chemical (St. Louis, MO).

RESULTS

Influence of forced metabolic oscillations in islets on insulin secretion in a control medium. The first series of experiments was performed in a medium containing a normal concentration of K^+ (4.8 mmol/l). In the absence of diazoxide, repetitive increases in the concentration of glucose (from 3 to 20 mmol/l) caused large oscillations of islet metabolism as shown by the oscillations in NAD(P)H fluorescence (Fig. 1*A*). The closure of K^+-ATP channels caused by glucose under these control conditions results in membrane depolarization and stimulation of Ca^{2+} influx (1–5). Thus, each stimulation with glucose induced a large increase in $[Ca^{2+}]_i$ and triggered a pulse of insulin secretion (Fig. 1*A*). When diazoxide was present in the medium to prevent closure of K^+-ATP channels and β-cell membrane depolarization, repetitive applications of 20 mmol/l glucose induced metabolic oscillations that were similar to those observed in the absence of diazoxide (Fig. 1*B*). These applications of high glucose simultaneously caused small decreases in $[Ca^{2+}]_i$ that are due to sequestration of the ion in the endoplasmic reticulum (32,33), but they did not affect basal insulin secretion (Fig. 1*B*). Metabolic oscillations are thus unable to trigger detectable oscillations of insulin secretion at basal $[Ca^{2+}]_i$.

Influence of forced metabolic oscillations in islets on insulin secretion in the presence of elevated $[Ca^{2+}]_i$. These experiments were performed in the presence of 30 mmol/l K^+ to raise $[Ca^{2+}]_i$ even in low glucose. In half of the experiments, diazoxide was added to the medium to prevent the small depolarization that glucose still produces under these conditions (7). Alternating between 3 and 20 mmol/l glucose again induced large oscillations in islet NAD(P)H fluorescence that were not affected by diazoxide (Fig. 2*A* and *B*). However, each rise in the glucose concentration was now accompanied by a limited decrease in $[Ca^{2+}]_i$ followed by a climb and complete correction on return to low glucose. These changes were more pronounced in the presence than in the absence of diazoxide and have previously been described and attributed to glucose-induced Ca^{2+} sequestration (7). A slow upward trend (2–3 nmol · l^{-1} · min^{-1}) characterized these long recordings of islet $[Ca^{2+}]_i$ in the presence of high K^+. A similar, albeit smaller, trend (1.5–2.0 nmol · l^{-1} · min^{-1}) has previously been observed in the continuous presence of 30 mmol/l K^+ and 3 mmol/l glucose with or without diazoxide (34).

Because of the elevation of $[Ca^{2+}]_i$ by high K^+, insulin secretion was already stimulated at 3 mmol/l glucose. The first

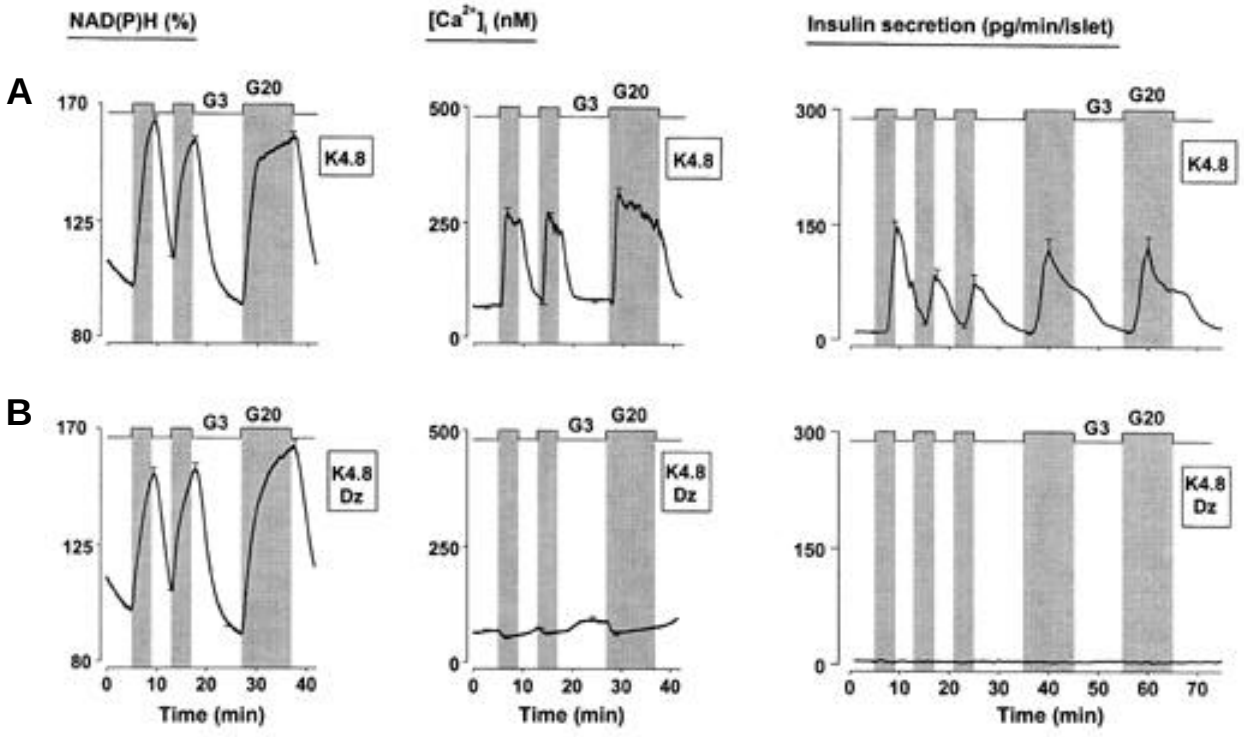

FIG. 1. Effects of forced metabolic oscillations on insulin secretion from mouse islets in a control medium. The left panels show the NAD(P)H fluorescence, the middle panels show $[Ca^{2+}]_i$ measured in fura-PE3–loaded islets, and the right panels show insulin secretion. *A*: The islets were perifused with a control medium containing 4.8 mmol/l K^+. To induce metabolic oscillations, the concentration of glucose (G) was raised intermittently from 3 to 20 mmol/l as indicated. *B*: Similar experiments were performed in the presence of 250 µmol/l diazoxide (Dz). Values are means ± SE for 13–25 individual islets from four to six distinct cultures ($[Ca^{2+}]_i$ and NAD(P)H) or for five to seven separate experiments with batches of 18 islets (insulin).

increase in the glucose concentration further augmented insulin secretion despite the fall in $[Ca^{2+}]_i$ (Fig. 2*A* and *B*). This potentiation reflects the K^+-ATP channel–independent action of glucose (7–11). Distinct oscillations of secretion corresponding to the glucose pulses were superimposed on this elevated secretory rate. Their amplitude was larger at low versus high frequencies of stimulation, and their nadir never approached the basal rate of secretion measured at low $[Ca^{2+}]_i$. Each oscillation also clearly lagged behind the period of glucose application, and this shift was more marked in the presence than in the absence of diazoxide (Fig. 2*A* and *B*).

In other experiments performed in the presence of high K^+, the glucose concentration was repetitively changed between 10 and 20 mmol/l. This resulted in distinct oscillations in islet NAD(P)H fluorescence that were smaller than those induced by the glucose excursions between 3 and 20 mmol/l but again were unaffected by diazoxide (Fig. 2*C* and *D*). These pulses of 20 mmol/l glucose did not have an effect on $[Ca^{2+}]_i$ in the presence of diazoxide and caused only small but consistent increases in the absence of diazoxide. The absence of a fall in $[Ca^{2+}]_i$ is probably explained by the fact that Ca^{2+} sequestration is already maximally stimulated by glucose concentrations <10 mmol/l (32). Under these conditions, the rate of insulin secretion increased on stimulation with 20 mmol/l glucose but did not clearly oscillate. Only when the glucose pulses were applied at the low frequency of 0.05/min did insulin secretion display oscillations of relatively small amplitude (Fig. 2*C* and *D*).

To define better the temporal correlations between secretory and other oscillations, insulin secretion was measured simultaneously with NAD(P)H or $[Ca^{2+}]_i$ during perifusion of single islets with high K^+ and diazoxide (Fig. 3). Alternating between 3 and 20 mmol/l glucose induced parallel oscillations of NAD(P)H fluorescence. Oscillations of insulin secretion were also evoked, but their ascending and descending phases were clearly delayed (Fig. 3*A*). During the first 2 min of high-glucose application, when metabolism rapidly augmented but $[Ca^{2+}]_i$ decreased, insulin secretion was not stimulated (Fig. 3*A*). Only when $[Ca^{2+}]_i$ started to increase slowly after the initial drop did insulin secretion increase. Strikingly, this increase continued during the $[Ca^{2+}]_i$ rebound and the fall in the metabolic signal that accompanied the return to low glucose. This time course points to a major influence of $[Ca^{2+}]_i$ changes. However, at the end of each stimulation with 20 mmol/l glucose, the rate of secretion was higher than during the preceding period in low glucose although $[Ca^{2+}]_i$ was lower.

The impact of metabolism on the action of $[Ca^{2+}]_i$ was also evidenced by experiments in which the changes in the glucose concentration were between 10 and 20 mmol/l (Fig. 3*B*). Under these conditions, $[Ca^{2+}]_i$ did not change, and small oscillations of insulin secretion more accurately followed the oscillations of the metabolic signal. Metabolic oscillations can thus induce oscillations of insulin secretion when β-cell $[Ca^{2+}]_i$ is elevated and displays no oscillations (i.e., through the K^+-ATP channel–independent pathway). However, these oscillations are variable between islets and are, on average, of small amplitude.

Influence of forced $[Ca^{2+}]_i$ oscillations in islets on insulin secretion in the presence of 10 mmol/l glucose. In both the absence and presence of diazoxide, the large $[Ca^{2+}]_i$ oscillations induced by repetitive depolariza-

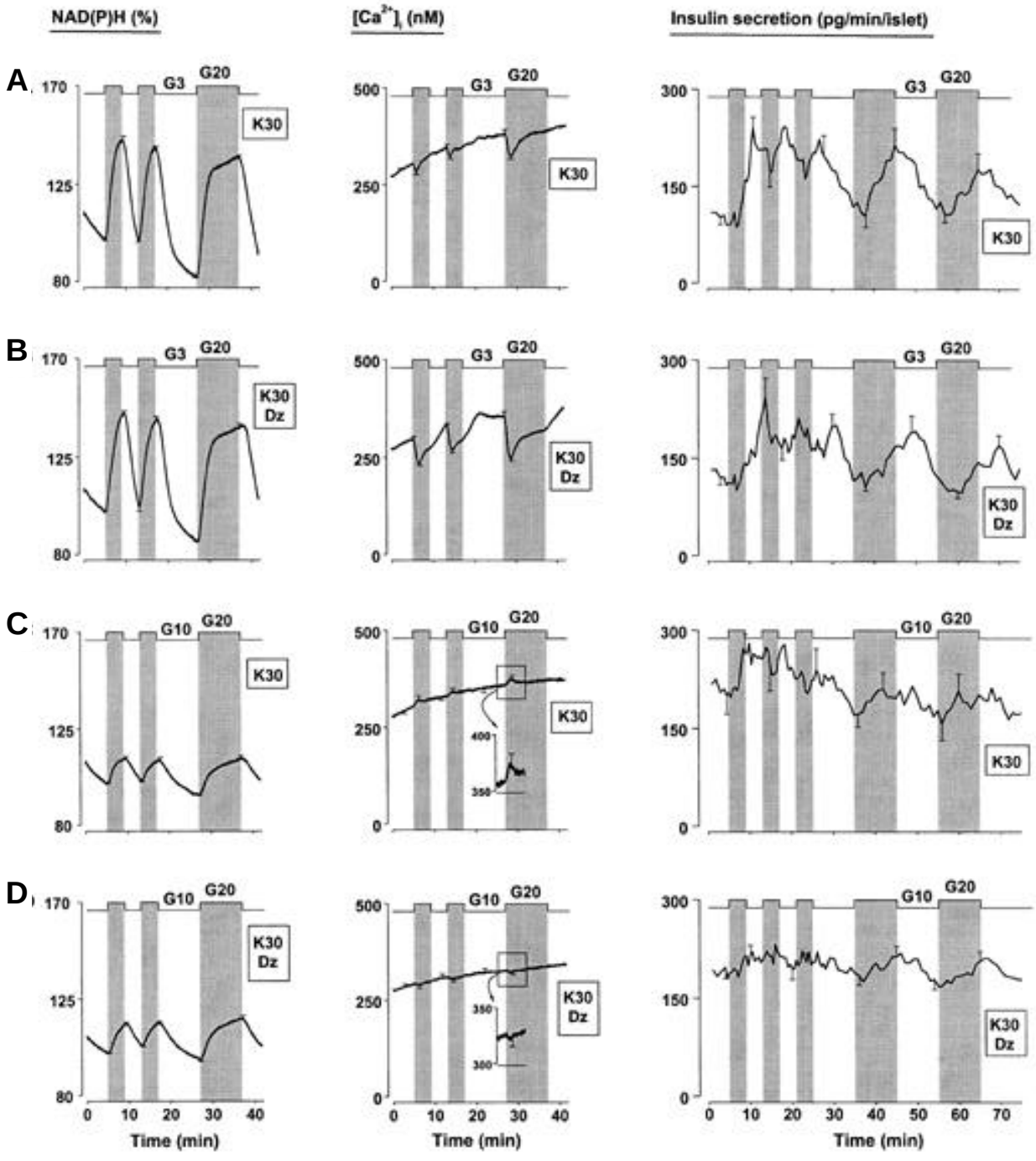

FIG. 2. Effects of forced metabolic oscillations on insulin secretion from mouse islets depolarized with 30 mmol/l K^+ (K30) in the absence or presence of 250 µmol/l diazoxide (Dz). The concentration of glucose was raised intermittently from 3 to 20 mmol/l (*A* and *B*) or from 10 to 20 mmol/l (*C* and *D*). Values are means ± SE for 14–27 individual islets from four to seven distinct cultures ($[Ca^{2+}]_i$ and NAD(P)H) or for six to eight separate experiments with batches of 18 islets (insulin).

tions with 30 mmol/l K^+ evoked small increases in islet NAD(P)H fluorescence and large oscillations of insulin secretion (Fig. 4). These changes in insulin secretion were not only larger but also were faster than those induced by alternating the glucose concentration in the presence of stable high $[Ca^{2+}]_i$ (compare Figs. 2 and 4). $[Ca^{2+}]_i$ oscillations are thus potent inducers of oscillations of insulin secretion, even when they are associated with only small metabolic oscillations.

Influence of sustained activation of PKA and PKC activity in islets on $[Ca^{2+}]_i$-induced insulin secretion. If Ca^{2+}-independent oscillations in the phosphorylation state of effector proteins are more important than $[Ca^{2+}]_i$ oscillations themselves to generate oscillations of insulin secretion, then the latter should no longer follow forced $[Ca^{2+}]_i$ changes during sustained activation of protein kinases.

Forskolin was used to stimulate the adenylate cyclase directly and produce a continuous activation of PKA from the

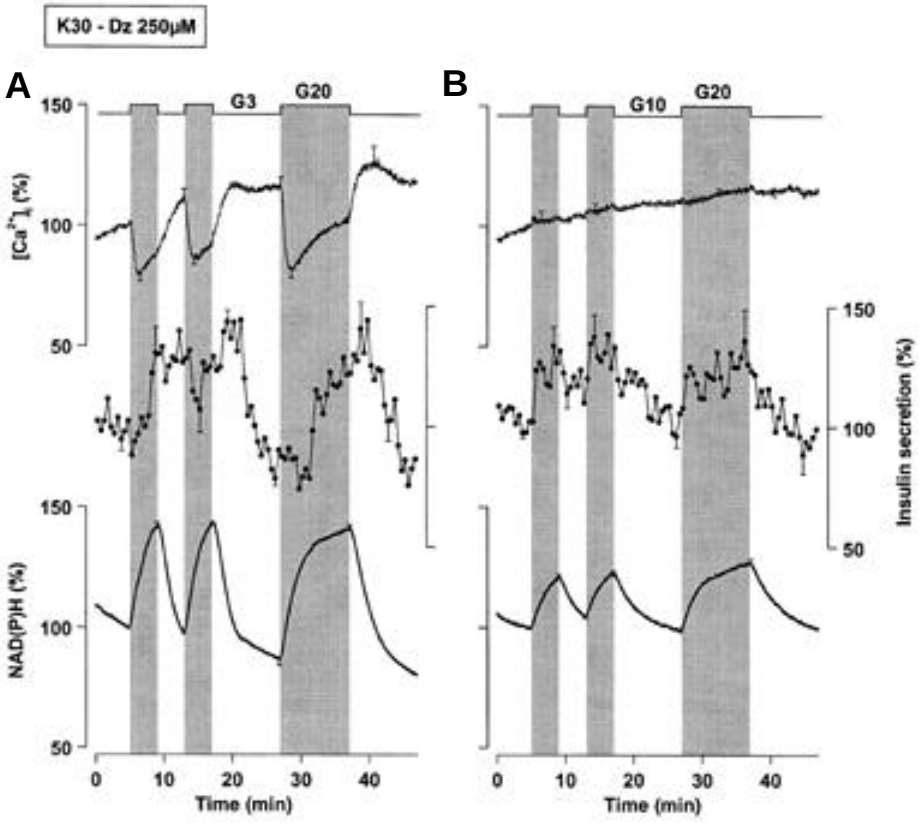

FIG. 3. Simultaneous measurements of insulin secretion and $[Ca^{2+}]_i$ or NAD(P)H fluorescence in single mouse islets during forced metabolic oscillations. The islets were depolarized with 30 mmol/l K^+ in the presence of 250 μmol/l diazoxide (Dz). The concentration of glucose was raised intermittently from 3 to 20 mmol/l (*A*) or from 10 to 20 mmol/l (*B*). All results are expressed as a percentage of the values measured within each experiment during the last minute preceding the first application of 20 mmol/l glucose. Values are means ± SE for eight experiments of insulin secretion, of which four were combined with $[Ca^{2+}]_I$, and four were combined with NAD(P)H measurement. The islets were obtained from four separate preparations.

elevated levels of cAMP (14,35). Repetitive depolarizations by 30 mmol/l K^+ triggered slightly larger $[Ca^{2+}]_i$ oscillations in the presence versus the absence of forskolin (Fig. 5*A*). This is ascribed to the potentiation by cAMP of Ca^{2+} influx through voltage-dependent Ca^{2+} channels (35,36). The imposed rhythm of insulin secretion was not perturbed by forskolin, but the amplitude of each oscillation was 2.5- to 4-fold larger than in control islets (Fig. 5*A*). Qualitatively similar results

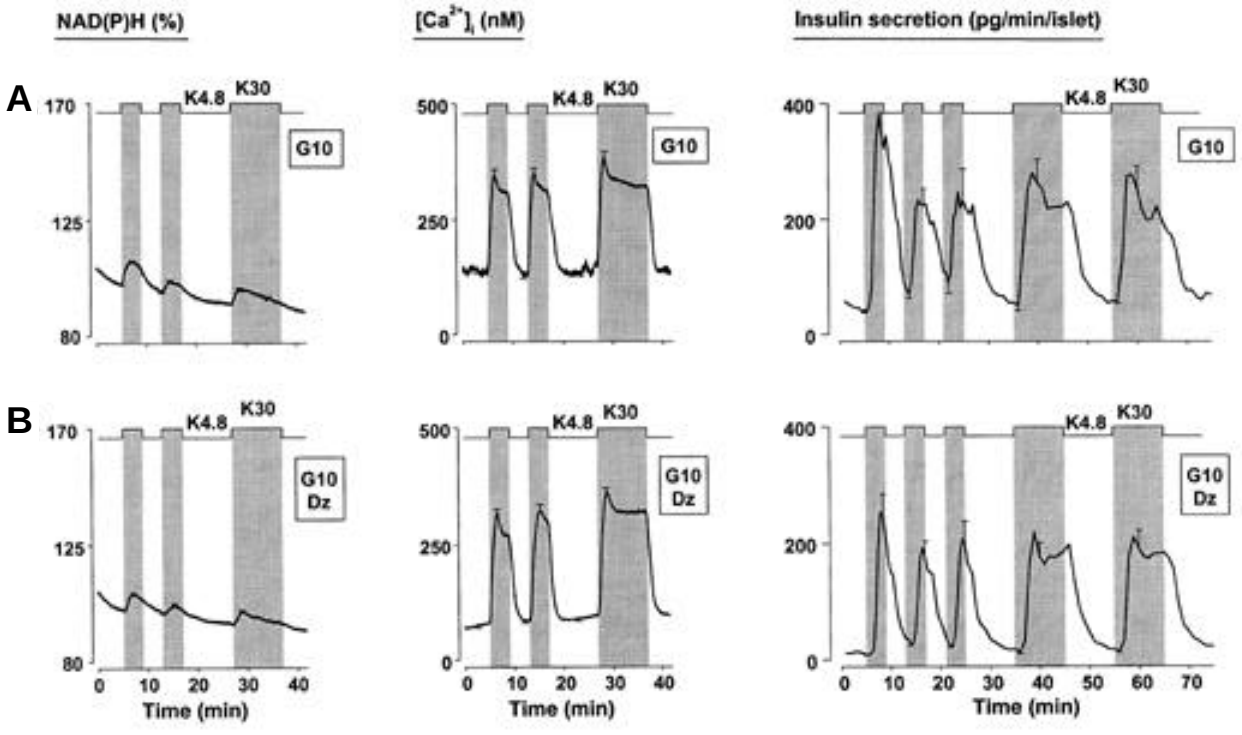

FIG. 4. Effects of forced $[Ca^{2+}]_i$ oscillations on insulin secretion from mouse islets stimulated with 10 mmol/l glucose (G10) in the absence or presence of 250 μmol/l diazoxide (Dz). The concentration of K^+ was raised intermittently from 4.8 to 30 mmol/l. Values are means ± SE for 14–23 individual islets from six to eight distinct cultures ($[Ca^{2+}]_i$ and NAD(P)H) or for six to nine separate experiments with batches of 18 islets (insulin).

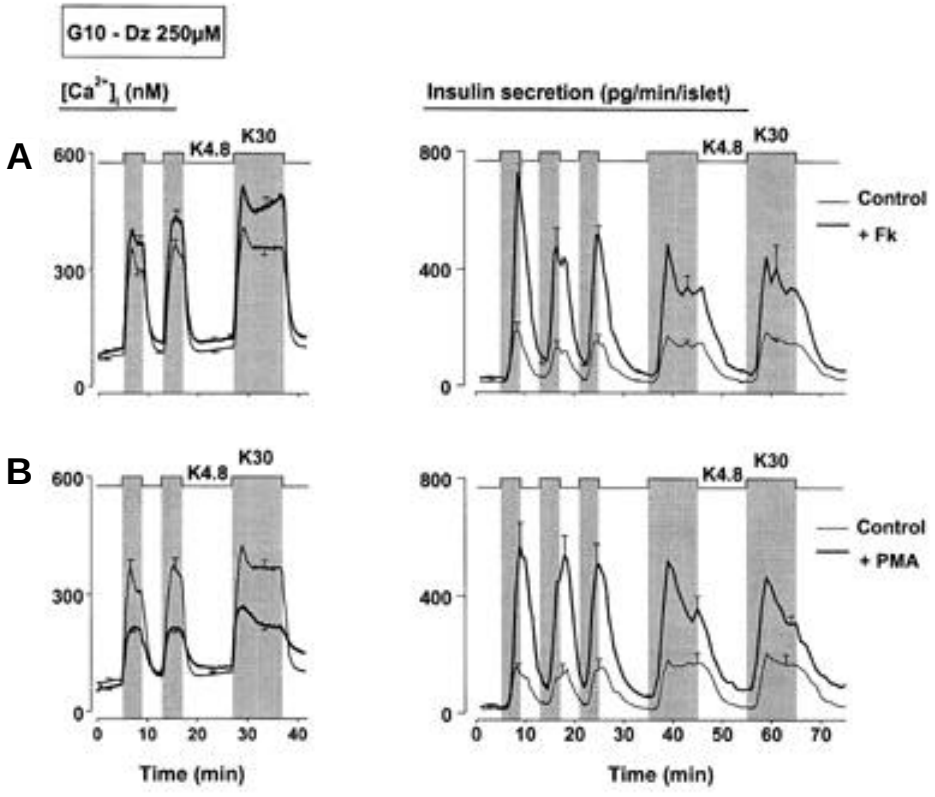

FIG. 5. Effects of forced $[Ca^{2+}]_i$ oscillations on insulin secretion from mouse islets during continuous activation of PKA and PKC. The experiments were carried out in the continuous presence of 10 mmol/l glucose and 250 µmol/l diazoxide (G10-Dz), and the islets were intermittently depolarized by increasing the concentration of K^+ from 4.8 to 30 mmol/l. *A*: Forskolin (Fk, 1 µmol/l) was used to raise cAMP and activate PKA. *B*: PMA (25 nmol/l) was used to activate PKC. Values are means ± SE for 15–20 individual islets from four to five different cultures ($[Ca^{2+}]_i$) and for four to five experiments with batches of 12 islets (insulin secretion).

were obtained with 1 mmol/l dibutyryl cAMP, except that the potentiation of insulin secretion was less than with 1 µmol/l forskolin (data not shown).

Direct continuous stimulation of PKC was achieved with 25 nmol/l PMA (12–14). The phorbol ester did not perturb the rhythm of $[Ca^{2+}]_i$ oscillations imposed by the repetitive depolarizations with high K^+ but markedly reduced their amplitude (Fig. 5*B*). This is unlikely to be explained by an inhibition of Ca^{2+} influx (37) but rather may be due to an acceleration of Ca^{2+} efflux (38). The oscillations of insulin secretion still followed the rhythm imposed by the $[Ca^{2+}]_i$ changes, but their magnitude was greatly increased by PMA (Fig. 5*B*).

These experiments clearly show that oscillations of $[Ca^{2+}]_i$ remain able to induce oscillations of insulin secretion even when PKA and PKC are continuously stimulated. The magnitude of the oscillations of secretion is increased under these conditions. This potentiation is not commensurate with the increase in $[Ca^{2+}]_i$ (PKA) or even accompanied by a decrease in $[Ca^{2+}]_i$ (PKC), which confirms that both protein kinases increase the effectiveness of Ca^{2+} on exocytosis (39,40).

DISCUSSION

The model. Insulin secretion is a Ca^{2+}-dependent and pulsatile process. Exocytosis of insulin granules can be stimulated by a rise in $[Ca^{2+}]_i$ or by an amplification of the Ca^{2+} action through changes in metabolism or protein kinase activity. Oscillations of insulin secretion could thus theoretically result from oscillations of either of these factors. This study evaluated whether this is possible by forcing each factor to oscillate while stabilizing or controlling the others as much as possible. Most experiments were carried out in the presence of high K^+ with or without diazoxide to prevent or minimize the effects of glucose on β-cell membrane potential and $[Ca^{2+}]_i$. Although several studies have shown that diazoxide does not alter glucose oxidation (41,42) and adenine nucleotide levels (28) in mouse islets, others have reported slight inhibition of mitochondrial function caused by the drug (43,44). Importantly, our measurements of NAD(P)H fluorescence did not show any untoward effects of the drug on metabolism, which could invalidate our conclusions.

$[Ca^{2+}]_i$ and metabolic oscillations were forced by repetitive applications of the appropriate stimulus. At least two frequencies of $[Ca^{2+}]_i$ oscillations have been identified in glucose-stimulated islets. They have periods of <30 s and of 3–5 min (19,45–47). Spontaneous metabolic oscillations have only rarely been described in β-cells or in whole islets steadily stimulated by glucose. Their period was 45 s for NAD(P)H fluorescence (48), 5.5 min for O_2 consumption (47), and 18 min for lactate release (49). We did not try to mimic any of these frequencies but rather elected to stimulate the islets at a rate that induced indisputably measurable $[Ca^{2+}]_i$ and metabolic oscillations. Otherwise, the failure to observe oscillations of secretion could have been ascribed to an absence of real metabolic oscillations. The concentration of glucose was thus alternated every 4 min (period of 8 min) because higher rates of application of high glucose did not induce reproducible well-defined oscillations of NAD(P)H fluorescence under conditions of sufficient $[Ca^{2+}]_i$ stability. Although $[Ca^{2+}]_i$ oscillations can easily be induced at a higher frequency (29), these were induced at the same rhythm as metabolic oscillations to permit valid comparison of the efficacy of the two signals.

One could argue that short-lived increases in insulin secretion after repetitive stimulations are predictable. Such a simplistic criticism would ignore recent reports emphasizing the contrary because of possible desensitization of the releasing process (50). This argument would also misconstrue the aim of our study, which was to determine whether the secretory response to one type of forced oscillations is influenced by the

experimental conditions and by the association with other types of oscillations.

Oscillations of metabolism and $[Ca^{2+}]_i$. Repetitive changes in the glucose concentration (3–20 mmol/l) in a control medium generated large metabolic and $[Ca^{2+}]_i$ oscillations that induced pulses of insulin secretion. When diazoxide was used to prevent $[Ca^{2+}]_i$ oscillations, the forced metabolic oscillations persisted, but the oscillations of secretion were abrogated or became so small that they were undetectable in our system. In this regard, our results contrast with those of one study that reported that insulin secretion from *ob/ob* mouse islets oscillates even when the medium contains no or only 3 mmol/l glucose with or without 400 µmol/l diazoxide (51). Because β-cell $[Ca^{2+}]_i$ was presumably low in these islets, the phenomenon was tentatively ascribed to spontaneous metabolic oscillations, but these were not identified, and their nature in the absence of exogenous fuel was unclear. Our observation that forced metabolic oscillations do not induce detectable oscillations of secretion at low $[Ca^{2+}]_i$ levels in normal mouse islets does not prove that $[Ca^{2+}]_i$ oscillations are necessary to induce pulsatile insulin release; our results simply indicate that $[Ca^{2+}]_i$ must be elevated.

The same repetitive glucose stimulations were, therefore, applied when islet $[Ca^{2+}]_i$ was steadily raised by high K^+. Large metabolic oscillations again occurred that were accompanied by an increase in insulin secretion despite a small decrease of $[Ca^{2+}]_i$. This increase in secretion was characterized by oscillations of limited amplitude. Their nadir did not reach basal rates of insulin secretion so that they remained superimposed on the high rate of secretion that was determined by the elevated $[Ca^{2+}]_i$ in low glucose. The time course of these insulin changes was clearly influenced by the small fluctuations of $[Ca^{2+}]_i$. Whenever $[Ca^{2+}]_i$ decreased, even to a limited extent, the increase in secretion was delayed despite the rapid increase in metabolism. Conversely, the rebound rises of $[Ca^{2+}]_i$ prevented secretion from immediately following the decrease in the metabolic signal that accompanied the return to low glucose. The asynchrony of two positive signals (rises in $[Ca^{2+}]_i$ and metabolism) and two negative signals (decreases in $[Ca^{2+}]_i$ and metabolism) explains why the oscillations of secretion lagged behind the glucose pulses and why they were better defined when the frequency of the stimulation was low. When the glucose concentration varied between 10 and 20 mmol/l only, the concentration of $[Ca^{2+}]_i$ remained stable, and the increases and decreases in insulin secretion more closely followed those of the metabolic signal. However, because the amplitude of the latter was small, the oscillations of secretion did not exceed 15–20%, which made them sometimes difficult to discern unless the frequency of stimulation was low. Altogether, these experiments establish that metabolic oscillations can induce oscillations of insulin in the presence of elevated stable $[Ca^{2+}]_i$ and that they effectively do so only when their amplitude is large or their frequency is low. These experiments also show that even small variations of $[Ca^{2+}]_i$ have a marked influence on these oscillations.

When the β-cell membrane was repetitively depolarized by high K^+ levels in the presence of a constant glucose concentration, large $[Ca^{2+}]_i$ oscillations occurred that were accompanied by relatively small but consistent increases in NAD(P)H fluorescence, which probably reflect activation of mitochondrial dehydrogenases by Ca^{2+} (26,27,48). However, each $[Ca^{2+}]_i$ oscillation resulted in a large, well-synchronized oscillation of secretion. Thus, $[Ca^{2+}]_i$ oscillations are better able to drive oscillations of secretion in the presence of only small metabolic oscillations than are larger metabolic oscillations in the absence of $[Ca^{2+}]_i$ oscillations. The K^+-ATP channel–independent pathway is thus able to mediate glucose-induced oscillations of insulin secretion but only with a lesser efficacy than the K^+-ATP channel–dependent pathway.

Oscillations of PKA and PKC. Oscillations of protein kinase activity may conceivably be induced by oscillations of metabolism and/or $[Ca^{2+}]_i$ (12–14), but whether they could occur independently of the latter is unclear. In the intact pancreas, neural connections may coordinate the oscillatory secretory behavior of all islets (52), but this does not necessary imply oscillations of protein kinase activities. In vivo extrinsic factors may be involved (53). The present study shows that the ability of $[Ca^{2+}]_i$ oscillations to drive pulsatile insulin secretion was not impaired by continuous activation of PKA and PKC, which indicates that primary oscillations of protein kinase activity (and of the phosphorylation state of effector proteins) are not prerequisite signals for oscillations of secretion. However, the oscillations of $[Ca^{2+}]_i$ may cyclically stimulate Ca^{2+}/calmodulin-dependent protein kinase and certain isoforms of PKC that are known to be involved in insulin secretion (12–14,54).

Conclusions. This study provides the first direct support for the hypothesis that oscillations of metabolism can induce small oscillations of insulin secretion independently of $[Ca^{2+}]_i$ oscillations. That oscillations of β-cell membrane potential and $[Ca^{2+}]_i$ occur during stimulation by glucose alone is well established (18–21). The temporal association of these spontaneous $[Ca^{2+}]_i$ oscillations with pulses of insulin secretion has been directly demonstrated. Thus we have no doubt that glucose can induce large oscillations of insulin secretion through the K^+-ATP channel–dependent pathway. The present results suggest that the K^+-ATP channel–independent pathway may underlie smaller oscillations. However, when spontaneous oscillations of secretion have been observed in the absence of $[Ca^{2+}]_i$ oscillations, the authors have postulated that metabolic oscillations were driving the system (22–25). The next necessary step will be to demonstrate the existence of metabolic oscillations and their direct association with the oscillations of secretion.

The results also indicate that oscillations of $[Ca^{2+}]_i$ in β-cells have the greatest power to control oscillations of insulin secretion. Although forced metabolic oscillations may also cyclically influence secretion, their regulatory effects are weaker or are characterized by a hysteresis that makes them unlikely drivers of fast oscillations. However, under physiological conditions, several oscillators may cooperate to induce oscillations of secretion. A possible scenario is that intrinsic or induced metabolic oscillations generate oscillations of membrane potential and hence of $[Ca^{2+}]_i$. The latter would then induce oscillations of exocytosis, the amplitude of which may be increased by the activation of kinases and by metabolism itself. The primum movens is the metabolic oscillation, but the minute-to-minute regulator is $[Ca^{2+}]_i$.

ACKNOWLEDGMENTS

This work was supported by the Interuniversity Poles of Attraction Program (P4/21), Belgian State Prime Minister's Office, Federal Office for Scientific, Technical, and Cultural

Affairs; by Grant 3.4552.98 from the Fonds de la Recherche Scientifique Médicale, Brussels; and by Grant 95/00-188 from the General Direction of Scientific Research of the French Community of Belgium. P.G. is Chercheur Qualifié from the Fonds National de la Recherche Scientifique, Brussels.

We are grateful to Myriam Nenquin for help with some of the experiments, to Luc Cnops and Fabien Knockaert for technical assistance, and to Stéphanie Roiseux for editorial help. We thank Dr. Jean-Christophe Jonas for suggestions and comments.

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