Signals and Pools Underlying Biphasic Insulin Secretion

Jean-Claude Henquin, Nobuyoshi Ishiyama, Myriam Nenquin, Magalie A. Ravier, and Jean-Christophe Jonas

Rapid and sustained stimulation of β-cells with glucose induces biphasic insulin secretion. The two phases appear to reflect a characteristic of stimulus-secretion coupling in each β-cell rather than heterogeneity in the time-course of the response between β-cells or islets. There is no evidence indicating that biphasic secretion can be attributed to an intrinsically biphasic metabolic signal. In contrast, the biphasic rise in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by glucose is important to shape the two phases of secretion. The first phase requires a rapid and marked elevation of $[Ca^{2+}]_i$ and corresponds to the release of insulin granules from a limited pool. The magnitude of the second phase is determined by the elevation of $[Ca^{2+}]_i$, but its development requires production of another signal. This signal corresponds to the amplifying action of glucose and may serve to replenish the pool of granules that are releasable at the prevailing $[Ca^{2+}]_i$. The species characteristics of biphasic insulin secretion and its perturbations in pathological situations are discussed. *Diabetes* 51 (Suppl. 1):S60–S67, 2002

Glucose exerts both concentration- and time-dependent effects in β-cells. Upon stimulation by an abrupt and sustained increase in the ambient glucose concentration, insulin secretion occurs following a biphasic time course. The secretion rate initially accelerates markedly before slowing down (first phase), and eventually increases again at a slower rate or stabilizes depending on the preparation and the species (second phase). The biphasic pattern of insulin secretion was first clearly described in vitro, in the isolated and perfused rat pancreas (1) and in perifused rat islets (2). A similar biphasic time course characterizes the increase in plasma insulin concentration that a rapid and sustained elevation of the plasma glucose concentration induces in normal human subjects (3–5).

Several mechanisms might explain biphasic insulin secretion. The numerous islets composing the endocrine pancreas could be functionally heterogeneous, some of them being responsible for the first phase and others for the second phase (6). This hypothesis is refuted by the observation of biphasic insulin secretion from single islets (7–10). Another possibility is that, within each islet, different β-cells preferentially secrete during first or second phase. This is difficult to test because single β-cells respond poorly to glucose alone. However, there is no evidence that two populations of β-cells, sorted according to their glucose responsiveness, display distinct secretory dynamics (11). Moreover, β-cell coupling increases response homogeneity (12). If one accepts that the two phases of insulin secretion are not the expression of intra-islet β-cell heterogeneity, two major mechanisms can be envisaged: they are known as the "storage-limited model" and the "signal-limited model" (13,14). According to the "storage-limited model," each phase of secretion corresponds to the release, by a constant signal, of a distinct pool of insulin granules, the notion of "pool" corresponding to geographically or functionally distinct granules (15,16). According to the "signal-limited model," the biphasic response could be the result of a single biphasic stimulatory signal or of the sum of signals with different dynamics (17,18). These two models are not mutually exclusive and could coexist.

Glucose stimulates insulin secretion by generating triggering and amplifying signals in β-cells (19). The triggering pathway involves a now well-characterized sequence of events: metabolism of glucose by oxidative glycolysis, increase in the ATP/ADP ratio, closure of ATP-sensitive K^+ channels, membrane depolarization, opening of voltage-operated Ca^{2+} channels, Ca^{2+} influx, rise in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$), and activation of the exocytotic machinery. The amplifying pathway also depends on glucose metabolism but does not involve a further increase in $[Ca^{2+}]_i$; it serves to amplify the efficacy of Ca^{2+} on exocytosis of insulin granules through biochemical mechanisms that remain incompletely identified (19).

A biphasic metabolic signal? Owing to the critical role of glucose metabolism for stimulus-secretion coupling, one could envisage that the biphasic pattern of insulin secretion is determined by an intrinsically biphasic change of β-cell metabolism elicited by the sudden rise in glucose concentration. Unfortunately, not many biochemical events lend themselves to monitoring with the adequate time resolution. Reduced pyridine nucleotides (NADH and NADPH) increase in glucose-stimulated islets. Biochemical measurements at selected time points in batches of rat islets have suggested that this increase is biphasic (20), and continuous recording of the autofluorescence of NAD(P)H in rat β-cells has shown that the response is often biphasic (21). In mouse islets cultured under conditions favoring their flattening, hence permitting studies in one cell layer, the NAD(P)H fluorescence increase produced by glucose was faster in cytoplasm than mitochondria (22); however, this did not result in a clearly biphasic change of the global signal. In intact single mouse islets

From the Unité d'Endocrinologie et Métabolisme, University of Louvain School of Medicine, UCL 55.30, B-1200 Brussels, Belgium.
Address correspondence and reprint requests to henquin@endo.ucl.ac.be.
Accepted for publication 21 June 2001.
$[Ca^{2+}]_i$, cytoplasmic Ca^{2+} concentration.
The symposium and publication of this article have been made possible by an unrestricted educational grant from Servier, Paris.

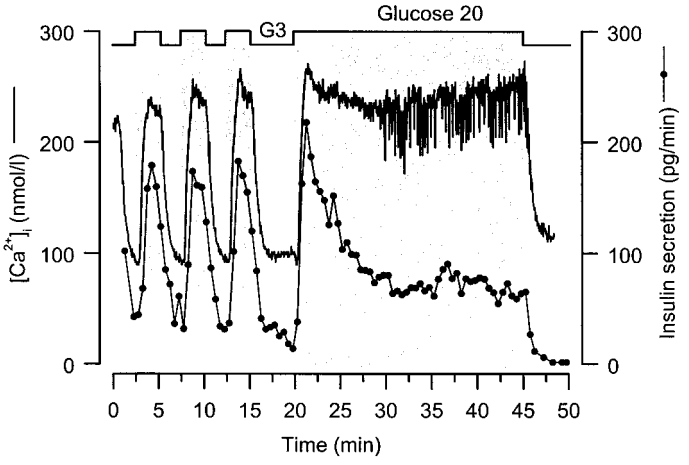

FIG. 1. Simultaneous measurements of $[Ca^{2+}]_i$ and insulin secretion in a single mouse islet. The islet was studied after 1 day of culture in RPMI medium containing 10 mmol/l glucose and incubation for 2 h in the presence of 2 μmol/l fura PE3 acetoxymethylester. The concentration of glucose (G) was changed between 3 and 20 mmol/l, as indicated.

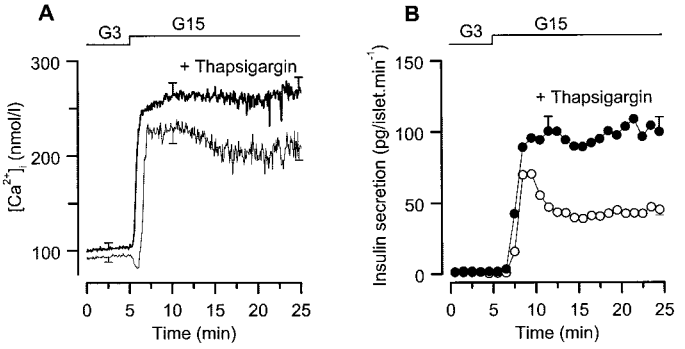

FIG. 2. Influence of thapsigargin, an inhibitor of the Ca^{2+}-ATPases of the endoplasmic reticulum, on glucose-induced $[Ca^{2+}]_i$ and insulin secretion changes in mouse islets. The measurements were done separately in different systems. $[Ca^{2+}]_i$ was measured in individual islets loaded with fura PE3 during 2 h of incubation with or without 1 μmol/l thapsigargin. Insulin secretion was measured with groups of 25 islets preincubated with or without thapsigargin, and without fura PE3. In both systems, the concentration of glucose (G) was raised from 3 to 15 mmol/l, as indicated. *A*: Means ± SE for 8–10 individual islets. *B*: Means ± SE for five experiments.

stimulated by glucose, the increase has consistently been found to be monophasic (23–25). In perifused mouse islets stimulated by different glucose concentrations, oxygen consumption increased monophasically although simultaneously measured insulin secretion displayed its typical biphasic pattern (26). In single mouse islets, a biphasic increase in oxygen consumption has sometimes been detected, but this pattern is thought to be secondary to concomitant changes in $[Ca^{2+}]_i$ (27). Overall, there is no convincing evidence indicating that biphasic secretion can be attributed to a primary (i.e., Ca^{2+}-independent) biphasic change of a metabolic signal.

A biphasic change in $[Ca^{2+}]_i$. Before $[Ca^{2+}]_i$ could be directly measured in β-cells, indirect methods were used to identify the mechanisms leading to the rise of Ca^{2+} that was assumed to trigger secretion. This led to contradictory proposals that first phase of glucose-induced insulin secretion was caused by Ca^{2+} influx from the extracellular medium (28) or by Ca^{2+} mobilization from intracellular pools (29). There is now general consensus that Ca^{2+} influx through voltage-dependent Ca^{2+} channels is necessary. In mouse islets, glucose-induced rise in $[Ca^{2+}]_i$ displays a biphasic time course: an initial sustained elevation is followed by oscillations or by a lower plateau, depending on the glucose concentration (8,24,30,31). This pattern is similar to that of the electrical activity induced by glucose in β-cells (17). The correspondence is not surprising because the electrical activity reflects Ca^{2+} influx through voltage-dependent Ca^{2+} channels.

The obvious question is thus whether the biphasic time course of glucose-induced insulin secretion could not simply be the consequence of a biphasic triggering signal. Figure 1 shows simultaneous changes in $[Ca^{2+}]_i$ and insulin secretion in the same mouse islet. Several stimulations with 20 mmol/l glucose for periods of 2.5 min every 5 min evoked similar $[Ca^{2+}]_i$ increases and simultaneous pulses of insulin secretion. When the period of stimulation by high glucose was extended to 25 min, $[Ca^{2+}]_i$ declined after the initial peak and rapid oscillations appeared, resulting in a lower mean $[Ca^{2+}]_i$. Again, the initial $[Ca^{2+}]_i$ rise triggered a rapid and large secretory response, but insulin secretion subsequently decreased before stabilizing. This biphasic pattern with a lower, fairly stable second phase is typical for the mouse pancreas (32,33). The triggering signal $[Ca^{2+}]$ and the response (insulin secretion) thus show parallel time courses. However, the difference between the secretory rate at peak and plateau is clearly greater than the difference in $[Ca^{2+}]_i$ at the same times. This suggests that the correspondence between $[Ca^{2+}]_i$ and insulin is better temporally than quantitatively, in other words that other factors may be involved.

Does a change in the $[Ca^{2+}]_i$ pattern affect the secretory pattern? If the time-course of the glucose-induced $[Ca^{2+}]_i$ change in β-cells influences that of the secretory response, its alteration should perturb the two phases of insulin secretion. Stimulation of control islets with 15 mmol/l glucose caused a transient decrease in $[Ca^{2+}]_i$ followed by an increase whose biphasic pattern is here attenuated by the averaging of traces obtained in several islets (Fig. 2*A*). Mean $[Ca^{2+}]_i$ between 7 and 11 min was about 30 nmol/l higher than during the second phase. Test islets were pretreated with thapsigargin, a drug that blocks Ca^{2+} pumping into the endoplasmic reticulum. As shown previously (34), thapsigargin suppressed the small $[Ca^{2+}]_i$ decrease that normally precedes the first-phase rise (Fig. 2*A*). After thapsigargin pretreatment, glucose-induced $[Ca^{2+}]$ elevation was faster and larger than in control islets, and was monophasic. Insulin secretion, measured in separate islets, was increased by thapsigargin and no longer showed the characteristic biphasic pattern (Fig. 2*B*). The monophasic secretion of insulin induced by glucose thus followed the altered time course of the $[Ca^{2+}]_i$ change. It is possible, however, that the decline in secretion normally associated with second phase in the mouse was here prevented because $[Ca^{2+}]_i$ was increased to supranormal concentrations.

Glucose triggers biphasic insulin secretion only when its concentration is rapidly raised as a "square-wave." A "slow-ramp" increase induces gradually larger secretion without a first phase (15,35). The experiments shown in Fig. 3 were performed to determine whether $[Ca^{2+}]$ is the signal that must change rapidly for glucose-stimulated β-cells to develop first-phase insulin secretion. In a first

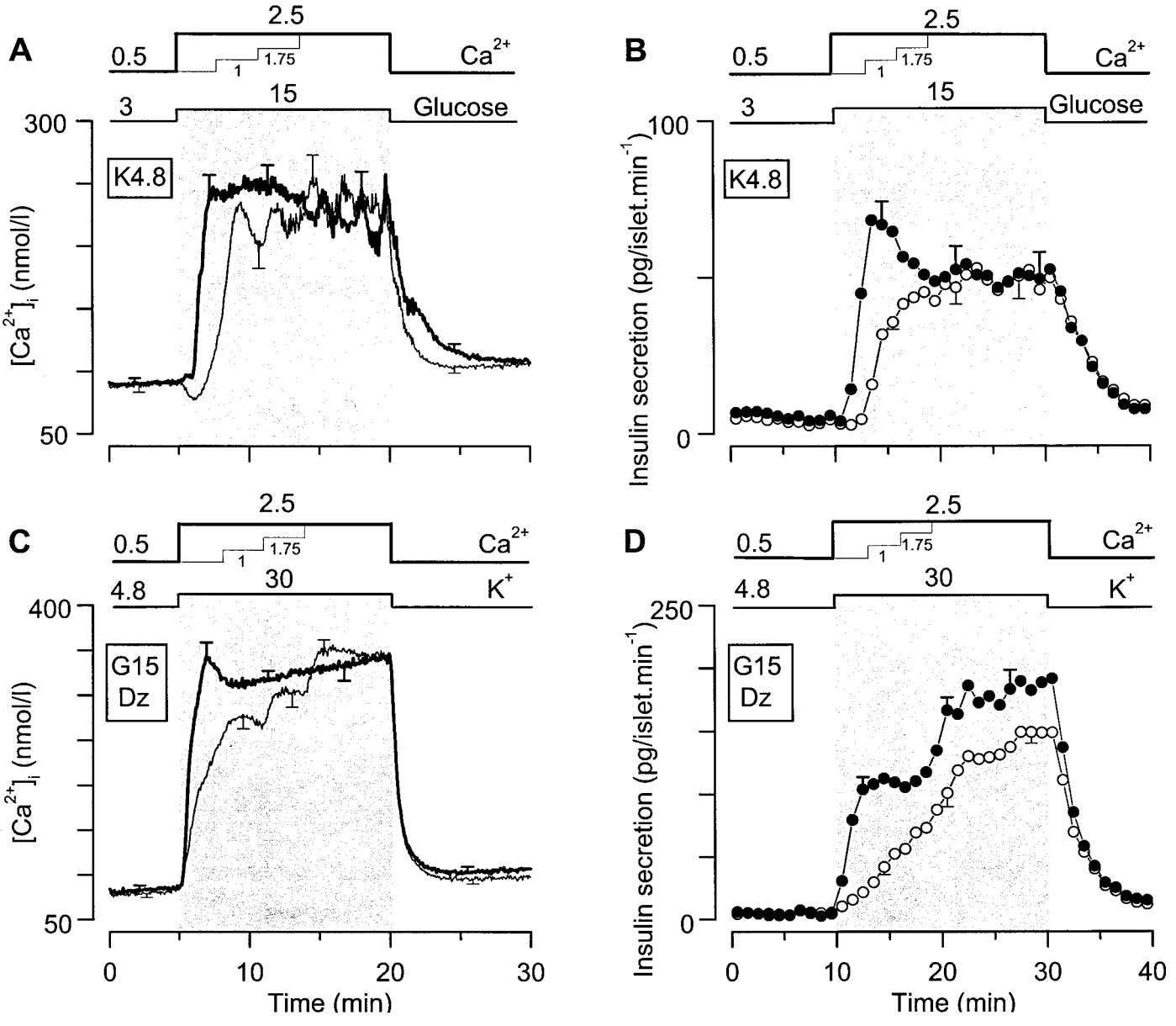

FIG. 3. Influence of a delay in the $[Ca^{2+}]_i$ rise induced by glucose or high K^+ on the pattern of insulin secretion by mouse islets. The measurements were done separately in different systems, as in Fig. 2. *A* and *B*: The medium contained 4.8 mmol/l K^+ throughout, whereas the glucose concentration was changed between 3 and 15 mmol/l as indicated. *C* and *D*: The medium contained 15 mmol/l glucose (G) and 100 μmol/l diazoxide (Dz) throughout, whereas the concentration of K^+ was changed between 4.8 and 30 mmol/l as indicated. *A*, *B*, *C*, and *D*: The concentration of Ca^{2+} in the medium was increased from 0.5 to 2.5 mmol/l stepwise (thin lines and ○) or in a single step (thick lines and ●). Values are means ± SE for 10 islets (*A*) or six islets (*C*) and for five experiments (*B* and *D*).

series (Fig. 3*A* and *B*), mouse islets were stimulated by a rapid rise of the glucose concentration from 3 to 15 mmol/l. In the control group, the concentration of extracellular Ca^{2+} was abruptly raised from 0.5 to 2.5 mmol/l concomitantly with the glucose stimulation. This resulted in a rapid increase in $[Ca^{2+}]_i$, followed by a slight decrease (averaging attenuates the biphasic pattern), and in a biphasic secretion of insulin. In the test group, the increase in extracellular $[Ca^{2+}]_i$ was delayed and stepwise (0.5–1.0 to 1.75–2.5 mmol/l), which resulted in a retarded and slower, but eventually similar increase in $[Ca^{2+}]_i$ (Fig. 3*A*). However, insulin secretion induced by glucose was monophasic, without first phase (Fig. 3*B*).

A slightly different approach was followed in the experiments shown in Fig. 3*C* and *D*. The glucose concentration was maintained at 15 mmol/l throughout, but its effects on $[Ca^{2+}]_i$ and insulin secretion were prevented by diazoxide. The triggering signal of secretion was thus provided by 30 mmol/l K^+. In the control group, the concentration of extracellular Ca^{2+} was increased from 0.5 to 2.5 mmol/l at the time of K^+ stimulation. This resulted in a rapid increase in $[Ca^{2+}]_i$, with an initial peak followed by a small decrease, then a progressive rise (Fig. 3*C*). Insulin secretion was rapidly stimulated following a peculiar biphasic time course, with a larger second than first phase (Fig. 3*D*). This pattern has previously been observed using a similar experimental paradigm (36). In the test group, the increase in extracellular Ca^{2+} was delayed and stepwise, which resulted in a slower increase in $[Ca^{2+}]_i$ (Fig. 3*C*). As a result, K^+-induced insulin secretion was progressive,

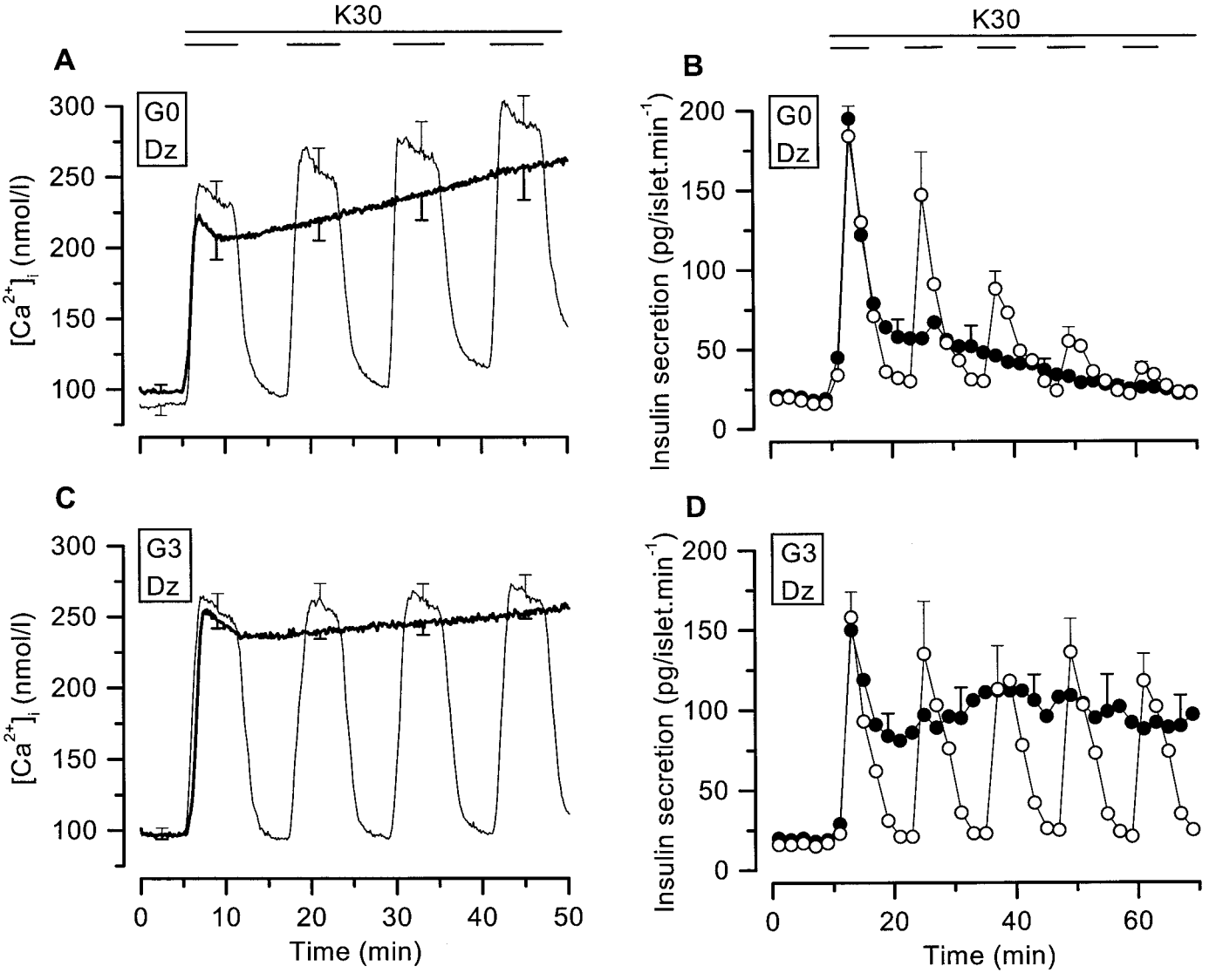

FIG. 4. Effects of a continuous or intermittent $[Ca^{2+}]_i$ rise on insulin secretion by mouse islets. The medium contained no or 3 mmol/l glucose (G) and 250 μmol/l diazoxide (Dz) throughout. The concentration of K^+ was continuously (thick lines and ●) or intermittently (thin lines and ○) increased from 4.8 to 30 mmol/l. Values are means ± SE for eight individual islets (*A* and *C*) and four experiments (*B* and *D*).

without the initial rapid phase. Altogether these data show that, whether the glucose concentration is rapidly increased or is constantly elevated, first-phase insulin secretion does not occur if $[Ca^{2+}]_i$ does not rapidly increase. The rapid change in glucose metabolism accompanying the glucose stimulation is not a sufficient signal.

Evidence for pools of releasable granules. In the absence of glucose and presence of diazoxide, continuous depolarization of mouse islets with 30 mmol/l K^+ caused a sustained and steadily increasing elevation of $[Ca^{2+}]_i$ (Fig. 4*A*). It also induced a monophasic secretion of insulin, characterized by a large initial peak followed by a progressive and almost complete return to basal values (Fig. 4*B*). A similar pattern of insulin secretion was observed when permeabilized islet or HIT cells were stimulated by a constant elevation of free Ca^{2+} (37,38). When the stimulation of intact islets with high K^+ was not continuous but intermittent (periods of 6 min every 12 min), similar increases in $[Ca^{2+}]_i$ were produced by each pulse of K^+ (Fig. 4*A*), but the magnitude of the insulin response decreased with the repetition of the stimulation (Fig. 4*B*). Interestingly, the area under the two insulin curves was similar, suggesting that only a finite pool of granules can be secreted during stimulation by $[Ca^{2+}]$ in the absence of glucose.

When similar experiments were repeated in presence of 3 mmol/l glucose, continuous depolarization caused a sustained, only slowly increasing elevation of $[Ca^{2+}]_i$, and repetitive stimulations caused similar peaks of $[Ca^{2+}]_i$ (Fig. 4*C*). The presence of this low glucose concentration was sufficient to prevent the loss of Ca^{2+} efficacy on insulin secretion. During continuous depolarization, the immediate secretory response was followed by a partial decrease and a sustained second phase (Fig. 4*D*). During repetitive stimulation, the amplitude of the insulin responses slightly decreased between the first and second or third $[Ca^{2+}]_i$ pulse, but then remained stable (Fig. 3*D*). Similar observations have been made with single or groups of islets when $[Ca^{2+}]_i$ was intermittently raised in the presence of higher glucose concentrations (39,40).

The initial, rapid $[Ca^{2+}]_i$ rise appears to trigger release of a limited pool of granules and glucose seems to ensure refilling of this pool. This conclusion is in full agreement with that reached by experiments measuring membrane capacitance as a marker of exocytosis in single β-cells (41). A step increase in $[Ca^{2+}]_i$ elicits a biphasic exocytotic response consisting in an initial, rapid, ATP-independent phase and a second, slower, sustained, ATP-dependent phase. The question is whether first-phase insulin secretion in intact cells corresponds to the emptying of this pool of readily releasable granules. The size of "first-phase pool" can be estimated by measuring the dynamics of glucose-induced insulin secretion and the insulin content in the same islets. In perifused mouse islets, about 0.45% of total insulin is secreted during the first 5 min of stimulation by 15 mmol/l glucose (42). A similar size of the readily releasable pool was calculated by capacitance measurements in single cells stimulated by a train of depolarizations, but the release was completed over a considerably shorter time (10 s) (41). In contrast, strong stimulations (2 μmol/l Ca^{2+}) of single β-cells in the whole cell mode may release up to 25% of the insulin content in about 2 min (43). Such an enormous rate of secretion never occurs in intact β-cells and is irrelevant to studies of the pools of granules involved in glucose-induced biphasic insulin secretion.

The second phase of insulin secretion. The observation that inhibitors of protein synthesis selectively depressed the second phase of glucose-induced insulin secretion by the perfused rat pancreas (1) originally led to the proposal that this phase requires synthesis of new insulin granules. This interpretation was subsequently shown not to be correct (44,45). The consequences of protein synthesis inhibition in β-cells are time-dependent, the first mechanism causing inhibition of insulin secretion being the disappearance of short half-life proteins involved in the amplifying action of glucose (46).

According to the signal-limited model, two opposite effects of glucose might interact to shape the second phase: a time-dependent inhibition and a time-dependent potentiation (18). The concept of a time-dependent inhibitory signal is partly based on the following observations. When the perfused rat pancreas is stimulated by two brief (5 min every 10 min) elevations of the glucose concentration the second burst of insulin secretion is smaller than the first one as if an inhibitory signal was attenuating the response, and causing the trough between the two phases during sustained stimulation (18). The concept is not challenged by the experiments shown in Fig. 1, because the $[Ca^{2+}]$ and insulin changes evoked by the first application of high glucose were not measured. In other experiments in which the two changes were measured in distinct islets, successive stimulations with 20 mmol/l glucose (4 min every 8 min) induced similar $[Ca^{2+}]$ rises but the second insulin response was smaller than the first one (40). A similar behavior was observed upon repetitive stimulation with high K^+ in the presence of glucose and diazoxide (Fig. 4*C* and *D*) (39,40). If such an inhibitory signal exists, it is not an impairment of the glucose-induced or K^+-induced $[Ca^{2+}]_i$ rise. An alternative but disputed (47) interpretation of time-dependent inhibition would be that provision of insulin granules to the releasable pool is not rapid enough to refill the pool to the same extent as before the first stimulation.

According to the store-limited model, transfer of insulin granules from a reserve to a releasable pool is necessary for sustained second phase. Figure 4*A* and *B* clearly shows that an elevation of $[Ca^{2+}]_i$ in β-cells is not a sufficient signal to induce sustained insulin secretion in the absence of glucose (48) or other fuels (36). As little as 3 mmol/l glucose is sufficient to permit development of a second phase during depolarization with high K^+ (Fig. 4*C* and *D*). The magnitude of this second phase increases with the concentration of glucose, and may even become larger than the first one above 10 mmol/l glucose (Fig. 3*D*), although the concentration of $[Ca^{2+}]_i$ does not increase (36). This phenomenon corresponds to the amplifying action of glucose, i.e., the increase by the sugar of the efficacy of Ca^{2+} on the secretory process (19). It is probably equivalent to the time-dependent potentiating action of glucose (18). Whereas its essential contribution to the second phase of glucose-induced insulin secretion is widely accepted (19,36,49,50), its underlying mechanisms are still incompletely understood. Mobilization of insulin granules from a reserve to a releasable pool (41) is plausible. This replenishment may involve physical movement thanks to the cytoskeleton (51–54) or change in granule properties (16).

Physiologically, the second phase of glucose-induced insulin secretion is determined by the amplitude of the $[Ca^{2+}]_i$ rise in β-cells and the amplification of the action of $[Ca^{2+}]$ on the secretory machinery (19). It is important to bear in mind that these two effects of glucose can normally not be dissociated during steady-state stimulation. Changes in the glucose concentration affect second phase by changing both signals.

Species characteristics of biphasic insulin secretion. All experimental results presented in the figures of this article have been obtained with isolated mouse islets in which second-phase insulin response to glucose is usually flat and lower than the peak of the first phase. A similar pattern is also found with the perfused mouse pancreas (32,33). In contrast, in the rat pancreas and in isolated rat islets, the second phase of glucose-induced insulin secretion increases, often well above the peak of the first-phase, for up to 1–2 h of stimulation. This difference in the magnitude of second phase between the two species has variably been attributed to a greater production of cyclic AMP (55) or expression of distinct isoforms of protein kinase C (56) in the rat β-cell. These proposals implicitly mean that the response to the triggering signal is more strongly potentiated in the rat than the mouse. However, it should also be emphasized that the depolarization and subsequent rise in $[Ca^{2+}]_i$ induced by glucose in rat islets are different from those in the mouse. In single rat β-cells, glucose-induced $[Ca^{2+}]_i$ increase is extremely heterogenous: slowly rising, transient, monophasic or biphasic (57–59). In intact rat islets, a short lasting increase has been reported (60), but more recent experiments indicate that the $[Ca^{2+}]$ elevation is sustained, with a slightly higher initial response than the persistent plateau (31). The major difference with the mouse islet appears to be the absence of $[Ca^{2+}]_i$ oscillations under steady-state stimulation.

In normal human subjects, a rapid marked and sus-

tained increase in plasma glucose concentration, achieved by glucose perfusion, induces a biphasic elevation of plasma insulin concentration (61–63). After the initial peak a delayed increase often occurs, a pattern resembling that observed in the perfused rat pancreas. However, when human islets are isolated and perifused, the two phases of glucose-induced insulin secretion occur following a pattern similar to that of mouse islets (64–68) (J.-C.H., unpublished data). Studies of glucose-induced $[Ca^{2+}]_i$ changes in human islets have also shown that the biphasic "mouse-like" pattern predominates over the "rat-like" pattern (69).

Perturbations of biphasic insulin secretion. All conditions interfering with the rapid increase in $[Ca^{2+}]_i$ that glucose normally produces in β-cells may be expected to impair first-phase insulin secretion. This may be achieved experimentally, or occurs as a side-effect during treatment with inhibitors of voltage-dependent Ca^{2+} channels or activators of K^+-ATP channels. Less obviously, this may also be produced by agents that maintain β-cells depolarized in a glucose-independent manner.

When islets are stimulated by a high concentration of K^+ or sulfonylurea, $[Ca^{2+}]_i$ is elevated and insulin secretion is stimulated even in low glucose. A rise in the glucose concentration (e.g., from 3 to 15 mmol/l) does not elevate $[Ca^{2+}]_i$ further but strongly increases secretion. However, this increase in secretion is slightly delayed, progressive and monophasic, without typical first phase (40,42,70). It was anticipated that islets from mice without functional K^+-ATP channels in their β-cells would respond to glucose as do islets from normal mice after blockade of these channels with sulfonylureas, i.e., essentially with a second phase mediated by the amplifying action of glucose (19). This was, at least qualitatively, the case after inactivation of the sulfonylurea receptor (SUR 1) subunit (71) but not after inactivation of the pore forming (Kir 6.2) subunit (72). In the latter model, glucose was practically ineffective on insulin secretion in vitro. Persistent hyperinsulinemic hypoglycemia of infancy is often caused by an inactivating mutation of SUR 1; unexpectedly, however, these infants may rapidly release insulin after intravenous glucose injection (73). Thus, no coherent picture emerges from these preliminary studies of artificial or spontaneous models of K^+-ATP channel inactivation. Further studies are needed to understand stimulus-secretion coupling in these β-cells chronically subjected to high $[Ca^{2+}]_i$.

Impaired first-phase insulin secretion in response to glucose is an early sign of β-cell dysfunction in type 2 diabetic patients (3,74). Because agents like tolbutamide or arginine remain effective (75), the hypothesis that the pool of releasable granules is empty can be rejected. It is more likely that glucose is unable to raise $[Ca^{2+}]_i$ rapidly in these diseased β-cells.

CONCLUSIONS

The first phase of glucose-induced insulin secretion requires a rapid and marked elevation of $[Ca^{2+}]_i$ in β-cells, and corresponds to the release of granules from a limited pool. Although this pool is not emptied within 5 min of glucose stimulation, sustained secretion (second phase) requires production of a signal other than the $[Ca^{2+}]_i$ rise. This second signal corresponds to the amplifying action of glucose and may serve to replenish the pool of granules that are releasable at the prevailing $[Ca^{2+}]_i$. Whether physical translocation of granules, or change in granule properties is involved, remains open. The magnitude and the time-course of the triggering signal ($[Ca^{2+}]_i$) are also important to shape the biphasic pattern of the secretory response. This is also clearly demonstrated by the synchrony between the oscillations of β-cell $[Ca^{2+}]_i$ and insulin secretion, an aspect of the second phase of glucose-induced insulin secretion that is developed in other contributions to this volume.

REFERENCES

1. Curry DL, Bennett LL, Grodsky GM: Dynamics of insulin secretion by the perfused rat pancreas. *Endocrinology* 83:572–584, 1968
2. Lacy PE, Walker MM, Fink CJ: Perifusion of isolated rat islets in vitro: participation of the microtubular system in the biphasic release of insulin. *Diabetes* 21:987–998, 1972
3. Cerasi E, Luft R: The plasma insulin response to glucose infusion in healthy subjects and in diabetes mellitus. *Acta Endocrinol* 55:278–304, 1967
4. Porte D, Pupo AA: Insulin responses to glucose: evidence for a two pool system in man. *J Clin Invest* 48:2309–2319, 1969
5. Blackard WG, Nelson NC: Portal and peripheral vein immunoreactive insulin concentrations before and after glucose infusion. *Diabetes* 19:302–306, 1970
6. Aizawa T, Kaneko T, Komatsu M, Yajima H, Nagai M, Yamauchi K, Hashizume K: Functional heterogeneity of pancreatic islets (Abstract). *Diabetologia* 41 (Suppl. 1):A149, 1998
7. Gilon P, Shepherd RM, Henquin JC: Oscillations of secretion driven by oscillations of cytoplasmic Ca^{2+} as evidenced in single pancreatic islets. *J Biol Chem* 268:22265–22268, 1993
8. Bergsten P: Slow and fast oscillations of cytoplasmic Ca^{2+} in pancreatic islets correspond to pulsatile insulin release. *Am J Physiol* 268:E282–E287, 1995
9. Barbosa RM, Silva AM, Tomé AR, Stamford JA, Santos RM, Rosario LM: Control of pulsatile 5-HT/insulin secretion from single mouse panceatic islets by intracellular calcium dynamics. *J Physiol* 510:135–143, 1998
10. Ravier MA, Eto K, Jonkers FC, Nenquin M, Kadowaki T, Henquin JC: The oscillatory behaviour of pancreatic islets from mice with mitochondrial glycerol 3-phosphate dehydrogenase knockout. *J Biol Chem* 275:1587–1593, 2000
11. Van Schravendijk CFH, Kiekens R, Pipeleers DG: Pancreatic β cell heterogeneity in glucose-induced insulin secretion. *J Biol Chem* 267: 21344–21348, 1992
12. Jonkers FC, Henquin JC: Measurements of cytoplasmic Ca^{2+} in islet cell clusters show that glucose rapidly recruits β-cells and gradually increases the individual cell response. *Diabetes* 50:540–550, 2001
13. Cerasi E, Fick G, Rudemo M: A mathematical model for the glucose induced insulin release in man. *Eur J Clin Invest* 4:267–278, 1974
14. O'Connor MDL, Landahl H, Grodsky GM: Comparison of storage- and signal-limited models of pancreatic insulin secretion. *Am J Physiol* 238: R378–R389, 1980
15. Grodsky GM: A threshold distribution hypothesis for packet storage of insulin and its mathematical modeling. *J Clin Invest* 51:2047–2059, 1972
16. Daniel S, Noda M, Straub SG, Sharp GWG: Identification of the docked granule pool responsible for the first phase of glucose-stimulated insulin secretion. *Diabetes* 48:1686–1690, 1999
17. Meissner HP, Atwater IJ: The kinetics of electrical activity of beta cells in reponse to a "Square Wave" stimulation with glucose or glibenclamide. *Horm Metab Res* 8:11–16, 1976
18. Nesher R, Cerasi E: Biphasic insulin release as the expression of combined inhibitory and potentiating effects of glucose. *Endocrinology* 121:1017–1024, 1987
19. Henquin JC: Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes* 49:1751–1760, 2000
20. Malaisse WJ, Hutton JC, Kawazu S, Herchuelz A, Valverde I, Sener A: The stimulus-secretion coupling of glucose-induced insulin release: the links between metabolic and cationic events. *Diabetologia* 16:331–341, 1979
21. Pralong WF, Bartley C, Wollheim CB: Single islet beta-cell stimulation by nutrients: relationship between pyridine nucleotides, cytosolic Ca^{2+} and secretion. *EMBO J* 9:53–60, 1990
22. Patterson GH, Knobel SM, Arkhammar P, Thastrup O, Piston DW: Separa-

tion of the glucose-stimulated cytoplasmic mitochondrial NAD(P)H responses in pancreatic islet β cells. *Proc Natl Acad Sci U S A* 97:5203–5207, 2000
23. Panten U, Christians J, Kriegstein Ev, Poser W, Hasselblatt A: Effect of carbohydrates upon fluorescence of reduced pyridine nucleotides from perifused isolated pancreatic islets. *Diabetologia* 9:477–482, 1973
24. Gilon P, Henquin JC: Influence of membrane potential changes on cytoplasmic Ca^{2+} concentration in an electrically excitable cell, the insulin-secreting pancreatic B-cell. *J Biol Chem* 267:20713–20720, 1992
25. Duchen MR, Smith PA, Ashcroft FM: Substrate-dependent changes in mitochondrial function, intracellular free calcium concentration and membrane channels in pancreatic β-cells. *Biochem J* 294:35–42, 1993
26. Panten U, Lenzen S: Alterations in energy metabolism of secretory cells. In *Energetics of Secretion Responses*, Vol II. Akkerman J.-W. N., Ed. Boca Raton, Florida, CRC Press, 1988, p. 109–123
27. Jung S-K, Kauri LM, Qian W-J, Kennedy RT: Correlated oscillations in glucose consumption, oxygen consumption, and intracellular free Ca^{2+} in single islets of Langerhans. *J Biol Chem* 275:6642–6650, 2000
28. Henquin JC: Relative importance of extracellular and intracellular calcium for the two phases of glucose stimulated insulin release: studies with theophylline. *Endocrinology* 102:723–730, 1978
29. Wollheim CB, Kikuchi M, Renold AE, Sharp GWG: The roles of intracellular and extracellular Ca^{++} in glucose-stimulated biphasic insulin release by rat islets. *J Clin Invest* 62:451–458, 1978
30. Santos RM, Rosario LM, Nadal A, Garcia-Sancho J, Soria B, Valdeolmillos M: Widespread synchronous $[Ca^{2+}]_i$ oscillations due to bursting electrical activity in single pancreatic islets. *Pflügers Arch* 418:417–422, 1991
31. Antunes CM, Salgado AP, Rosario LM, Santos RM: Differential patterns of glucose-induced electrical activity and intracellular calcium responses in single mouse and rat pancreatic islets. *Diabetes* 49:2028–2038, 2000
32. Lenzen S: Insulin secretion by isolated perfused rat and mouse pancreas. *Am J Physiol* 236:E391–E400, 1979
33. Berglund O: Different dynamics of insulin secretion in the perfused pancreas of mouse and rat. *Acta Endocrinol* 93:54–60, 1980
34. Roe MW, Mertz RJ, Lancaster ME, Worley III JF, Dukes ID: Thapsigargin inhibits the glucose-induced decrease of intracellular Ca^{2+} in mouse islets of Langerhans. *Am J Physiol* 266:E852–E862, 1994
35. Sorenson RL, Lindell DV, Elde RP: Glucose stimulation of somatostatin and insulin release from the isolated, perfused rat pancreas. *Diabetes* 29:747–751, 1980
36. Gembal M, Detimary P, Gilon P, Gao ZY, Henquin JC: Mechanisms by which glucose can control insulin release independently from its action on ATP-sensitive K^+ channels in mouse B-cells. *J Clin Invest* 91:871–880, 1993
37. Jones PM, Persaud SJ, Howell SL: Ca^{2+}-induced insulin secretion from electrically permeabilized islets: loss of the Ca^{2+}-induced secretory response is accompanied by loss of Ca^{2+}-induced protein phosphorylation. *Biochem J* 285:973–978, 1992
38. Jonas JC, Li G, Palmer M, Weller U, Wollheim CB: Dynamics of Ca^{2+} and guanosine 5′-[gamma-thio]triphosphate action on insulin secretion from alpha-toxin-permeabilized HIT-T15 cells. *Biochem J* 301:523–529, 1994
39. Jonas JC, Gilon P, Henquin JC: Temporal and quantitative correlations between insulin secretion and stably elevated or oscillatory cytoplasmic Ca^{2+}in mouse pancreatic β cells. *Diabetes* 47:1266–1273, 1998
40. Ravier MA, Gilon P, Henquin JC: Oscillations of insulin secretion can be triggered by imposed oscillations of cytoplasmic Ca^{2+} or metabolism in normal mouse islets. *Diabetes* 48:2374–2382, 1999
41. Rorsman P, Eliasson L, Renström E, Gromada J, Barg S, Göpel S: The cell physiology of biphasic insulin secretion. *News Physiol Sci* 15:72–77, 2000
42. Anello M, Gilon, Henquin JC: Alterations of insulin secretion from mouse islets treated with sulphonylureas: perturbations of Ca^{2+} regulation prevail over changes in insulin content. *Br J Pharmacol* 127:1883–1891, 1999
43. Proks P, Eliasson L, Ammälä C, Rorsman P, Ashcroft FM: Ca^{2+}- and GTP-dependent exocytosis in mouse pancreatic β-cells involves both common and distinct steps. *J Physiol* 496:255–264, 1996
44. Sando H, Borg J, Steiner DF: Studies on the secretion of newly synthesized proinsulin and insulin from isolated rat islets of Langerhans. *J Clin Invest* 51:1476–1485, 1972
45. Sando H, Grodsky GM: Dynamic synthesis and release of insulin and proinsulin from perifused islets. *Diabetes* 22:354–360, 1973
46. Garcia-Barrado MJ, Ravier MA, Rolland JF, Gilon P, Nenquin M, Henquin JC: Inhibition of protein synthesis sequentially impairs distinct steps of stimulus-secretion coupling in pancreatic β cells. *Endocrinology* 142:299–307, 2001
47. Nesher R, Praiss M, Cerasi E: Immediate and time-dependent effects of glucose on insulin release: differential calcium requirements. *Acta Endocrinol* 117:409–416, 1988
48. Gembal M, Gilon P, Henquin JC: Evidence that glucose can control insulin release independently from its action on ATP-sensitive K^+ channels in mouse B-cells. *J Clin Invest* 89:1288–1295, 1992
49. Taguchi N, Aizawa T, Sato Y, Ishihara F, Hashizume K: Mechanism of glucose-induced biphasic insulin release: physiological role of adenosine triphosphate-sensitive K^+ channel-independent glucose action. *Endocrinology* 136:3942–3948, 1995
50. Aizawa T, Komatsu M, Asanuma N, Sato Y, Sharp GWG: Glucose action "beyond ionic events" in the pancreatic β-cell. *Trends Pharmacol Sci* 19:496–499, 1998
51. Malaisse WJ, Malaisse-Lagae F, Van Obberghen E, Somers G, Devis G, Ravazzola M, Orci L: Role of microtubules in the phasic pattern of insulin release. *Ann N Y Acad Sci* 253:630–652, 1975
52. Hisatomi M, Hidaka H, Niki I: Ca^{2+}/Calmodulin and cyclic 3,5′ adenosine monophosphate control movement of secretory granules through protein phosphorylation/dephosphorylation in the pancreatic β-cell. *Endocrinology* 137:4644–4649, 1996
53. Renström E, Eliasson L, Bokvist K, Rorsman P: Cooling inhibits exocytosis in single mouse pancreatic β-cells by suppression of granule mobilization. *J Physiol* 494:41–52, 1996
54. Easom RA: CaM kinase II: a protein kinase with extraordinary talents germane to insulin exocytosis. *Diabetes* 48:675–684, 1999
55. Ma YH, Wang J, Rodd GG, Bolaffi JL, Grodsky GM: Differences in insulin secretion between the rat and mouse: role of cAMP. *Eur J Endocrinol* 132:370–376, 1995
56. Zawalich WS, Zawalich KC: Regulation of insulin secretion by phospholipase C. *Am J Physiol* 271:E409–E416, 1996
57. Herchuelz A, Pochet R, Pastiels Ch, Van Praet A: Heterogeneous changes in $[Ca^{2+}]_i$ induced by glucose, tolbutamide and K^+ in single rat pancreatic β-cells. *Cell Calcium* 12:577–586, 1991
58. Theler J-M, Mollard P, Guérineau N, Vacher P, Pralong WF, Schlegel W, Wollheim CB: Video imaging of cytosolic Ca^{2+} in pancreatic β-cells stimulated by glucose, carbachol, and ATP. *J Biol Chem* 267:18110–18117, 1992
59. Hamakawa N, Yada T: Interplay of glucose-stimulated Ca^{2+} sequestration and acetylcholine-induced Ca^{2+} release at the endoplasmic reticulum in rat pancreatic β-cells. *Cell Calcium* 17:21–31, 1995
60. Martin F, Reig JA, Soria B: Secretagogue-induced $[Ca^{2+}]_i$ changes in single rat pancreatic islets and correlation with simultaneously measured insulin release. *J Mol Endocrinol* 15:177–185, 1995
61. Cerasi E: Mechanisms of glucose stimulated insulin secretion in health and in diabetes: some re-evaluations and proposals. *Diabetologia* 11:1–13, 1975
62. Luzi L, DeFronzo RA: Effect of loss of first-phase insulin secretion on hepatic glucose production and tissue glucose disposal in humans. *Am J Physiol* 257:E241–E246, 1989
63. Fritsche A, Stefan N, Hardt E, Schützenauer S, Häring H, Stumvoll M: A novel hyperglycaemic clamp for characterization of islet function in humans: assessment of three different secretagogues, maximal insulin response and reproducibility. *Eur J Clin Invest* 30:411–418, 2000
64. Ricordi C, Lacy PE, Finke EH, Olack BJ, Scharp DW: Automated method for isolation of human pancreatic islets. *Diabetes* 37:413–420, 1988
65. Davalli AM, Ricordi C, Socci C, Braghi S, Bertuzzi F, Fattor B, Di Carlo V, Pontiroli AE, Pozza G: Abnormal sensitivity to glucose of human islets cultured in a high glucose medium: partial reversibility after an additional culture in a normal glucose medium. *J Clin Endocrinol Metab* 72:202–208, 1991
66. Misler S, Barnett DW, Falke LC: Effects of metabolic inhibition by sodium azide on stimulus-secretion coupling in B cells of human islets of Langerhans. *Pflügers Arch* 421:289–291, 1992
67. Takei S, Teruya M, Grunewald A, Garcia R, Chan EK, Charles MA: Isolation and function of human and pig islets. *Pancreas* 9:150–156, 1994
68. Bertuzzi F, Saccomanno K, Socci C, Davalli AM, Taglietti MV, Berra C, Dalcin E, Monti LD, Pozza G, Pontiroli AE: Long-term in vitro exposure to high glucose increases proinsulin-like-molecules release by isolated human islets. *J Endocrinol* 158:205–211, 1998
69. Martin F, Soria B: Glucose-induced $[Ca^{2+}]_i$ oscillations in single human pancreatic islets. *Cell Calcium* 20:409–414, 1996
70. Sato Y, Anello M, Henquin JC: Glucose regulation of insulin secretion independent of the opening or closure of adenosine triphosphate-sensitive K^+ channels in β cells. *Endocrinology* 140:2252–2257, 1999
71. Seghers V, Nakazaki M, DeMayo F, Aguilar-Bryan L, Bryan J: SUR1 knockout mice. A model for K_{ATP} channel-independent regulation of insulin secretion. *J Biol Chem* 275:9270–9277, 2000
72. Miki T, Nagashima K, Tashiro F, Kotake K, Yoshitomi H, Tamamoto A, Gonoi T, Iwanaga T, Miyazaki J-I, Seino S: Defective insulin secretion and enhanced insulin action in K_{ATP} channel-deficient mice. *Proc Natl Acad Sci U S A* 95:10402–10406, 1998

73. Grimberg A, Ferry RJ, Kelly A, Koo-McCoy S, Polonsky K, Glaser B, Permutt MA, Aguilar-Bryan L, Stafford D, Thornton PS, Baker L, Stanley CA: Dysregulation of insulin secretion in children with congenital hyperinsulinism due to sulfonylurea receptor mutations. *Diabetes* 50:322–328, 2001
74. Pfeifer MA, Halter JB, Porte D Jr: Insulin secretion in diabetes mellitus. *Am J Med* 70:579–588, 1981
75. Ward WK, Beard JC, Halter JB, Pfeifer MA, Porte D Jr: Pathophysiology of insulin secretion in non-insulin-dependent diabetes mellitus. *Diabetes Care* 7:491–502, 1984