Original Article

In Vivo and In Vitro Glucose-Induced Biphasic Insulin Secretion in the Mouse

Pattern and Role of Cytoplasmic Ca^{2+} and Amplification Signals in β-Cells

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The mechanisms underlying biphasic insulin secretion have not been completely elucidated. We compared the pattern of plasma insulin changes during hyperglycemic clamps in mice to that of glucose-induced insulin secretion and cytosolic calcium concentration ($[Ca^{2+}]_c$) changes in perifused mouse islets. Anesthetized mice were infused with glucose to clamp blood glucose at 8.5 (baseline), 11.1, 16.7, or 30 mmol/l. A first-phase insulin response consistently peaked at 1 min, and a slowly ascending second phase occurred at 16.7 and 30 mmol/l glucose. Glucose-induced insulin secretion in vivo is thus biphasic, with a similarly increasing second phase in the mouse as in humans. In vitro, square-wave stimulation from a baseline of 3 mmol/l glucose induced similar biphasic insulin secretion and $[Ca^{2+}]_c$ increases, with sustained and flat second phases. The glucose dependency (3–30 mmol/l) of both changes was sigmoidal with, however, a shift to the right of the relation for insulin secretion compared with that for $[Ca^{2+}]_c$. The maximum $[Ca^{2+}]_c$ increase was achieved by glucose concentrations, causing half-maximum insulin secretion. Because this was true for both phases, we propose that contrary to current concepts, amplifying signals are also implicated in first-phase glucose-induced insulin secretion. To mimic in vivo conditions, islets were stimulated with high glucose after being initially perifused with 8.5 instead of 3.0 mmol/l glucose. First-phase insulin secretion induced by glucose at 11.1, 16.7, and 30 mmol/l was decreased by ~ 50%, an inhibition that could not be explained by commensurate decreases in $[Ca^{2+}]_c$ or in the pool of readily releasable granules. Also unexpected was the gradually ascending pattern of the second phase, now similar to that in vivo. These observations indicated that variations in prestimulatory glucose can secondarily affect the magnitude and pattern of subsequent glucose-induced insulin secretion. *Diabetes* 55:441–451, 2006

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$[Ca^{2+}]_c$, cytosolic calcium concentration; K_{ATP} channel, ATP-sensitive K^+ channel.

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Biphasic insulin secretion is the normal response of β-cells to a rapid and sustained increase in glucose concentration (1,2). The first phase corresponds to a prompt and marked, but transient (4–8 min), increase in the secretory rate. It is followed by a decrease to a nadir and a sustained flat or gradually increasing second phase that lasts as long as the glucose stimulation is applied. This peculiar pattern is observed in vitro, when a perfused pancreas or perifused islets are subjected to square-wave stimulation with high glucose (2,3), and characterizes the increase in plasma insulin concentration in human subjects during a hyperglycemic clamp (4,5).

First-phase insulin secretion is important for achieving optimal interstitial insulin concentrations (6) and suppressing hepatic glucose production (5,7). Although plasma glucose changes during meals are not rapid or large enough to elicit clear biphasic insulin secretion, the first phase reflects the ability of healthy β-cells to rapidly respond to a glucose challenge (8). Impairment of the first phase of glucose-induced insulin secretion has long and repeatedly been recognized as an early sign of β-cell dysfunction in type 2 diabetic patients (1,4,9–11). Recent studies have also shown that minimal elevations in fasting plasma glucose, within the conventionally considered normal range, is accompanied by a diminution of first-phase insulin secretion (12,13).

The possible mechanisms underlying the two phases of glucose-induced insulin secretion have been previously reviewed (14–18). Both the sequential release of distinct pools of insulin granules and the successive implication of distinct signals have been emphasized in models that can be reconciled. The first phase is currently ascribed to a rapid, ATP-sensitive K^+ channel (K_{ATP} channel)–dependent increase in β-cell cytosolic calcium concentration ($[Ca^{2+}]_c$) that triggers exocytosis of a small pool of readily releasable granules. The second phase requires both continuous production of the triggering signal (elevation of $[Ca^{2+}]_c$) and amplification of the action of Ca^{2+} on exocytosis by a K_{ATP} channel–independent mechanism. The molecular mechanisms of this amplification are still undefined (19) but could involve a refilling of the readily releasable pool by granule priming or translocation (17,18). It is generally agreed that the amplifying pathway is not involved in the first phase of glucose-induced

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insulin secretion, but this issue has not been directly tested.

It is well known that the kinetics of in vitro insulin secretion by the two most widely used model species are not identical. With either perifused islets or the perfused pancreas, second-phase insulin secretion is low and flat in the mouse (20–24) and gradually ascending in the rat (2,16,20,22,24). Because the plasma insulin concentration increases during the second phase of a hyperglycemic clamp in humans (5,16,25,26), the validity of the mouse model has sometimes been questioned (27); this issue is particularly problematic in a period of increasing use of transgenic mice or other mouse models of diabetes to understand the pathophysiology of type 2 diabetes.

In the present study we compared the pattern of plasma insulin changes during hyperglycemic clamps in mice to that of insulin secretion by perifused mouse islets during stimulation by high glucose. The changes in islet $[Ca^{2+}]_c$ were measured under similar conditions to assess the extent to which the pattern and amplitude of secretory responses depend on changes in the triggering signal or implicate amplifying signals.

RESEARCH DESIGN AND METHODS

The study was conducted in accordance with the guidelines for care and use of laboratory animals at both institutions.

Clamp technique in vivo. Female C57BL/6J mice (Taconic, Ry, Denmark) weighing 21.2 ± 0.3 g were used for the in vivo studies. The animals were kept on a 12 h:12 h light:dark schedule (lights on at 0600) and fed a standard pellet diet and tap water ad libitum. The experiments were performed in the late morning after animals were fasted for 4 h. The animals were anesthetized with an intraperitoneal injection of midazolam (0.4 mg/mouse; Dormicum; Hoffman-La Roche, Basel, Switzerland) and a combination of fluanison (0.9 mg/mouse) and fentanyl (0.02 mg/mouse; Hypnorm; Janssen, Beerse, Belgium). The anesthetics were again administered after 60 min. During the whole procedure, the animals were kept on a heating pad. After the anesthesia was delivered, the animals' right jugular vein and left carotid artery were catheterized. The venous catheter was used for the infusion of glucose or saline, and the arterial catheter was used for sampling.

At 30 min after the catheters were placed in the mice, D-glucose dissolved in saline was rapidly injected at a dose of 0.10, 0.25, or 0.75 g/kg; the total volume injected was 10 μl. One group of animals was injected with saline alone. Immediately thereafter, a continuous infusion of glucose (20 or 40 g/dl) or saline was begun at a rate of 1.0–2.0 μl/min. Plasma glucose was measured at 5-min intervals for 90 min by the glucose dehydrogenase technique with the use of a HemoCue analyzer (HemoCue, Ängelholm, Sweden). The rate of glucose infusion was adjusted according to the glucose values to maintain glucose levels at baseline, 11.1, 16.7, or 30 mmol/l. Blood samples were taken in heparinized tubes at t = 0, 1, 5, 20, 50, 70, and 90 min and immediately centrifuged; afterward the plasma was separated and stored at −20°C for analysis. The amount of blood taken from each mouse was 400–450 μl, which was partially compensated for by fluid infusion, so that the average net loss was ~225 μl. Plasma insulin was determined by a double-antibody radioimmunoassay using rat insulin as a standard (Linco Research, St. Charles, MO). Results are presented as means ± SE for 6–10 animals.

In vitro measurements of insulin secretion and islet $[Ca^{2+}]_c$. Female C57BL/6J mice (age 6–10 months; body weight 24.5 ± 0.4 g; n = 40) were obtained from Charles River Laboratories (Brussels, Belgium) or from local animal facilities. They had free access to food until they were killed by decapitation in the late morning (blood glucose: 7.00 ± 0.25 mmol/l). Islets were aseptically isolated by collagenase digestion of the pancreas (28) and, after being selected by hand, were cultured for ~18 h in RPMI 1640 medium (Invitrogen, Merelbeke, Belgium) containing 10% heat-inactivated FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 8.5 mmol/l glucose. This concentration of glucose was the same as mean plasma glucose at the start of the clamps.

The medium used for all in vitro experiments contained (in mmol/l): 120 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 24 $NaHCO_3$, and 5 HEPES with NaOH (pH 7.4). It was continuously gassed with a mixture of O_2/CO_2 (94:6) and was supplemented with 1 mg/ml BSA and appropriate glucose concentrations.

For insulin secretion measurements, batches of 20–30 cultured islets were placed in chambers of a dynamic perifusion system (29). All experiments began with a 40-min equilibration period, which was usually followed by a 120-min period of stimulation with high glucose. The effluent was collected at 2-min intervals during the last 10 min of the equilibration period, at 42-s intervals during the first 10 min of glucose stimulation, and then at 3-min intervals. (Small variations to this protocol occurred in special studies, as shown in the figures.) At the end of the experiments, the islets were recovered from the chambers, counted, and transferred in acid-ethanol for insulin extraction. Insulin in the effluent fractions and the diluted extracts was measured by radioimmunoassay using rat insulin as a standard and ethanol to precipitate bound insulin (30). The fractional insulin secretion rate was calculated as the percent of islet insulin content that was secreted per minute. The insulin content of the islets used for these experiments was 120 ± 4 ng/islet (n = 87). Results are thus presented as mean fractional insulin secretion rates for perifusions performed with the indicated number of different islet preparations. The peak insulin secretion rate during the first phase was determined as the mean of the two highest values between 2 and 9 min of glucose stimulation, and the mean insulin secretion rate during the first phase was computed over 7 min (2–9 min). The mean insulin secretion rate during the second phase was computed over 15 min (25–40 min, corresponding exactly to the period of $[Ca^{2+}]_c$ measurement) and 110 min (10–120 min, corresponding to the whole duration of the stimulation with glucose).

For $[Ca^{2+}]_c$ measurements, cultured islets were loaded with the Ca^{2+} indicator fura-PE3 (2 μmol/l, 2 h) in RPMI medium containing 8.5 mmol/l glucose. Loaded islets were then transferred (usually five to six at a time) into the perifusion chamber of a spectrofluorimetric system equipped with a camera, with which $[Ca^{2+}]_c$ was measured as previously described (31). Because of technical constraints, $[Ca^{2+}]_c$ experiments were shorter than insulin secretion experiments. Most of them began with a 30- to 35-min equilibration period (with recording during the last 20 min only) followed by a 40-min stimulation by high glucose. As previously reported (32), the apparent islet $[Ca^{2+}]_c$ slowly but steadily increases with time during perifusion with a medium containing a low or high glucose (or KCl) concentration. To avoid overestimation of $[Ca^{2+}]_c$ during the second versus the first phase of glucose stimulation, all values were corrected for an artifactual increase with a slope of 0.5 nmol · l^{-1} · min^{-1}. Glucose-induced $[Ca^{2+}]_c$ changes were measured in individual islets and averaged for presentation as mean traces ± SE. The peak $[Ca^{2+}]_c$ during the first phase was determined in each experiment as the mean of the seven consecutive highest values (corresponding to ~42 s) during the first 3 min of glucose stimulation (taking the highest values during the first 7 min did not alter the conclusions). The mean $[Ca^{2+}]_c$ during the first phase of glucose-induced $[Ca^{2+}]_c$ rise was computed over 7 min starting when $[Ca^{2+}]_c$ had increased at least 10 nmol/l above baseline (i.e., after the initial drop of $[Ca^{2+}]_c$, if any). The mean $[Ca^{2+}]_c$ during the second phase was computed over 15 min, between 25 and 40 min of glucose stimulation. In control experiments, stimulation with KCl (60 mmol/l) or arginine (10 mmol/l) during the first or second phase induced by 30 mmol/l glucose caused further increases in islet $[Ca^{2+}]_c$ (KCl $\gg$ arginine), showing that the recording capacity of the system was not saturated at glucose concentrations causing maximal $[Ca^{2+}]_c$ responses.

RESULTS

In vivo experiments. To reach the target of 8.5, 11.1, 16.7, or 30 mmol/l glucose in the four groups of mice, an initial glucose load of a different magnitude was given at t = 0. After the glucose peak, 1 min after this load, a variable glucose infusion was started to maintain the target over the 90-min study period. Figure 1*A* shows the infusion rate of glucose over the 90 min. Table 1 shows the magnitude of the glucose bolus, the resulting 1-min plasma glucose value, the mean glucose infusion rate over 90 min, and the resulting mean plasma glucose. Figure 1*B* and *C* shows plasma glucose and insulin levels during the clamps. It can be seen that glucose was maintained at fairly stable levels throughout the study period. The stability of plasma glucose in the control group receiving saline infusion indicated that blood sampling did not cause hemorrhagic stress in the mice. When glucose levels were increased, insulin levels peaked at 1 min and then declined to a nadir at 5 min, although glucose levels remained stably elevated. During the hyperglycemic clamp, an ascending second phase of plasma insulin was observed, which was of

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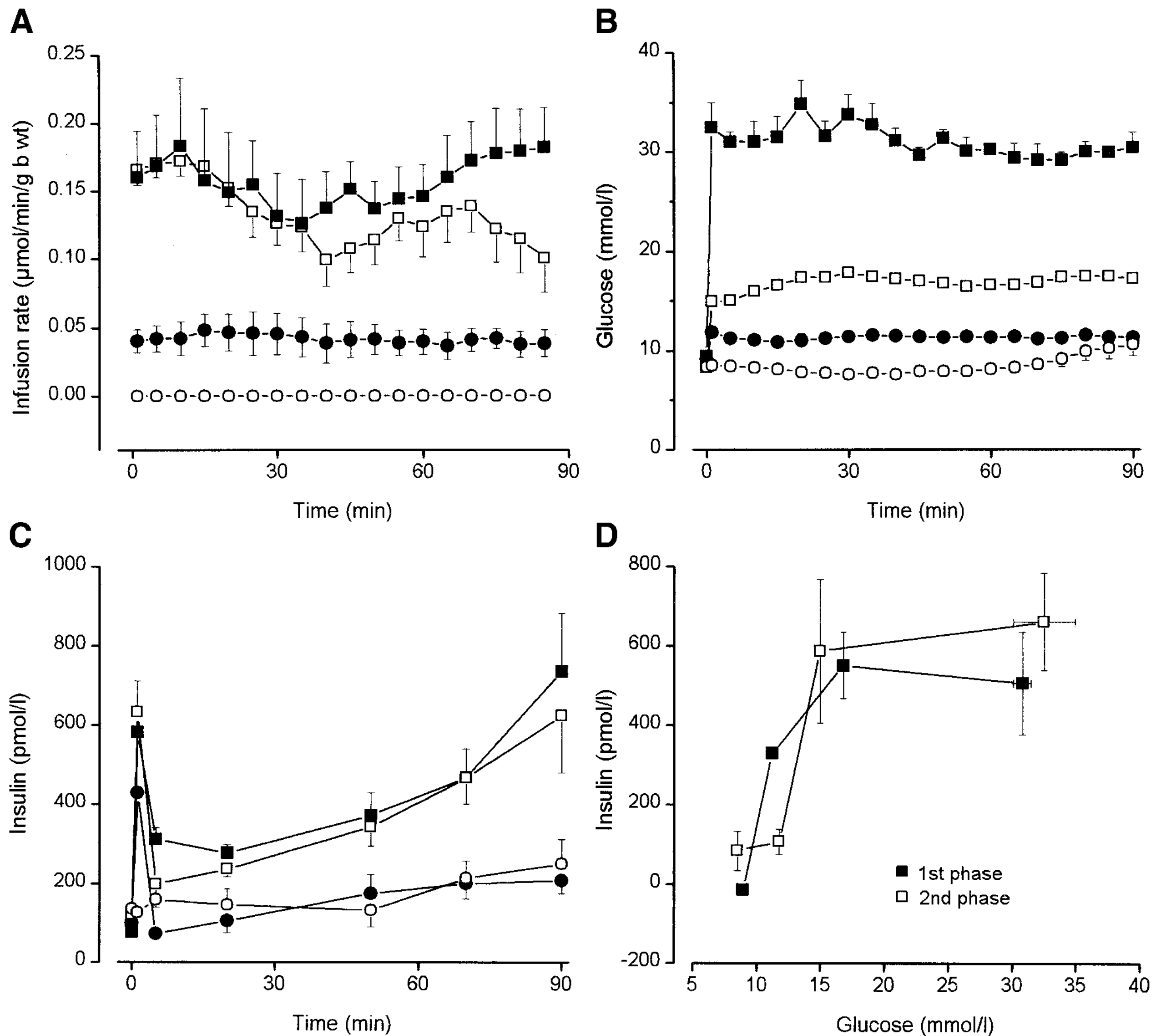

FIG. 1. Glycemic clamps in four groups of mice with the following target glucose concentrations: ○, baseline; ●, 11.1 mmol/l; □, 16.7 mmol/l; ■, 30 mmol/l. *A–C*: Glucose infusion rates (*A*), plasma glucose levels (*B*), and plasma insulin levels (*C*) are shown. *D*: First phase of insulin response to glucose (■, suprabasal 1-min insulin levels versus glucose levels at 1 min) and second phase of insulin response (□, suprabasal 90-min insulin levels versus mean glucose during the clamp). Data are means ± SE for 6–10 mice.

similar magnitude at 16.7 and 30 mmol/l glucose. In contrast, no second phase of plasma insulin rise occurred when glucose was kept at 11.1 mmol/l. Figure 1*D* shows the first and second phases of plasma insulin rise versus the glucose levels. The glucose threshold was lower for the first than the second phase, whereas maximal responses were similar.

In vitro experiments. Increasing the glucose concentration of the perifusion medium from 3 to 8.5 mmol/l induced triphasic changes in the islet $[Ca^{2+}]_c$ (Fig. 2, *inset*). An initial decrease below basal values was followed by a sharp peak increase and eventually by oscillations, the averaging of which attenuated the amplitude and resulted in a moderate mean elevation of the $[Ca^{2+}]_c$ above base-

TABLE 1
Amounts of glucose injected and blood glucose and plasma insulin levels during hyperglycemic clamps in mice

		Glucose injected (µmol/g)		Blood glucose (mmol/l)		Plasma insulin (pmol/l)	
Target glucose group	n	Bolus at 0 min	Infusion 1–90 min	1 min	Average 1–90 min	Suprabasal 1 min	Suprabasal 90 min
Baseline	9	0	0 ± 0	8.5 ± 0.3	8.9 ± 0.2	−15 ± 9	84 ± 49
11.1 mmol/l	6	0.6	3.7 ± 0.8	11.8 ± 0.5	11.2 ± 0.3	330 ± 33	107 ± 32
16.7 mmol/l	10	1.4	11.8 ± 1.0	15.0 ± 0.4	16.8 ± 0.6	551 ± 84	587 ± 181
30 mmol/l	6	4.1	14.1 ± 2.1	32.5 ± 2.4	30.8 ± 1.5	506 ± 129	660 ± 122

Data are means ± SE.

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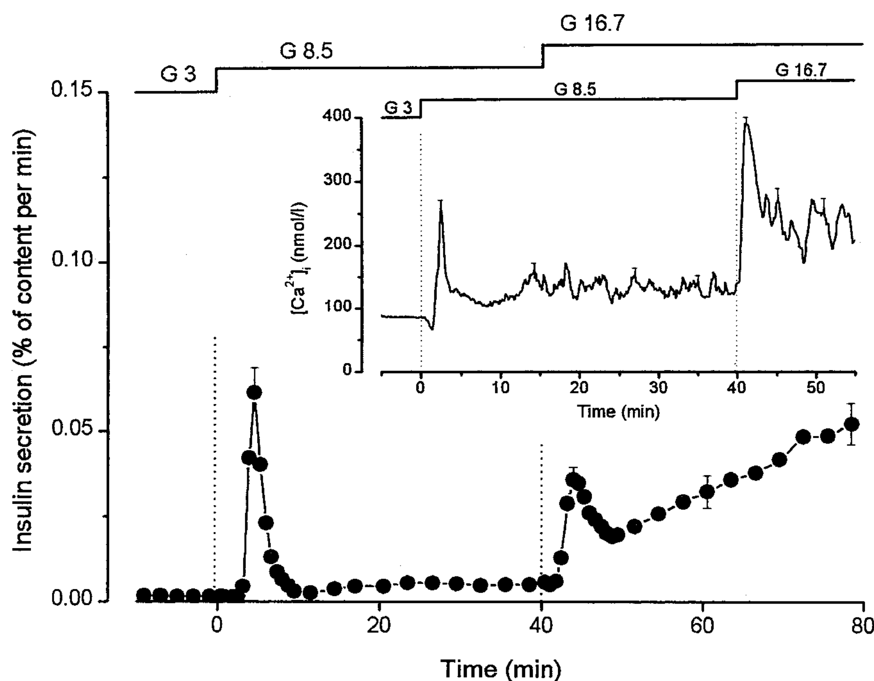

FIG. 2. Biphasic changes in insulin secretion and $[Ca^{2+}]_c$ (*inset*) in mouse islets stimulated by stepwise increases in the glucose (G) concentration of the perifusion medium. Data are means ± SE for eight experiments of insulin secretion and 36 islets from seven preparations for $[Ca^{2+}]_c$.

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line. In similar experiments, 8.5 mmol/l glucose induced biphasic insulin secretion characterized by a sharp and marked first phase and a sustained but small second phase (Fig. 2). A subsequent increase in glucose from 8.5 to 16.7 mmol/l evoked biphasic $[Ca^{2+}]_c$ and insulin responses. However, whereas the first-phase $[Ca^{2+}]_c$ was larger and longer than that initially produced by 8.5 mmol/l glucose, the first-phase insulin secretion was longer but clearly smaller. Moreover, the second-phase insulin secretion was not only larger but also showed an ascending pattern, which was unexpected for mouse islets.

In another series of experiments, the islets were stimulated by different glucose concentrations after being perifused for 40 min in 3 or 8.5 mmol/l glucose (Fig. 3*A–F*). Stimulation from 3 to 11.1, 16.7, or 30 mmol/l glucose evoked triphasic changes in the $[Ca^{2+}]_c$, with progressively smaller initial decreases and faster and longer first phases. Second phases of $[Ca^{2+}]_c$ changes were consistently characterized by oscillations, again largely masked by averaging (Fig. 3*B*, *D*, and *F*, *thick traces*). Under similar conditions of prestimulation with 3 mmol/l glucose, insulin secretion induced by the three glucose concentrations was biphasic, with a large and sharp first phase and a typically sustained and flat second phase (Fig. 3*A*, *C*, and *E*, ●).

The glucose dependency of the first and second phases of $[Ca^{2+}]_c$ and insulin secretion changes is summarized in Fig. 4. After initial perifusion in 3 mmol/l glucose (Fig. 4, ●), all relations were sigmoidal with, however, clear differences. The peak first-phase $[Ca^{2+}]_c$ already reached a maximum at 11.1 mmol/l glucose (Fig. 4*B*), whereas the mean first-phase $[Ca^{2+}]_c$ over 7 min (Fig. 4*D*) and mean second-phase $[Ca^{2+}]_c$ (Fig. 4*F*) increased up to 16.7 mmol/l glucose. Calculated K_m values were ~7.5 mmol/l glucose for the peak first phase and 11.0 mmol/l glucose for the mean first and second phases. In contrast, insulin secretion kept increasing up to 30 mmol/l glucose. Because it is known that insulin secretion is maximally stimulated by 30 mmol/l glucose in mouse islets (23), the following K_m values could be calculated: 10.7 mmol/l glucose for the peak first phase, 15 mmol/l glucose for the mean first phase, and 17 mmol/l glucose for the mean second phase. The K_m values for glucose-induced insulin secretion were thus consistently higher than those for the $[Ca^{2+}]_c$ rise.

When the experiments started with 8.5 instead of 3 mmol/l glucose, the prestimulatory $[Ca^{2+}]_c$ and insulin secretion rate were increased (Fig. 3*A–F*, ○ and *thin traces*). Stimulation with 11.1, 16.7, or 30 mmol/l glucose was followed by rapid, biphasic increases in $[Ca^{2+}]_c$ and insulin secretion (Fig. 3*A–F*). The higher prestimulatory glucose concentration (8.5 vs. 3 mmol/l) only had a minor influence on the subsequent glucose-induced $[Ca^{2+}]_c$ changes. No initial decrease occurred, and the first-phase increase started sooner (Fig. 3*B*, *D*, and *F*, *thin traces*). The peak of this first phase was not affected (Fig. 4*B*), but the mean first-phase $[Ca^{2+}]_c$ (over 7 min) was decreased slightly at 16.7 and 30 mmol/l glucose (Fig. 4*D*) because of a shorter duration (Fig. 3*D* and *F*). The mean second-phase $[Ca^{2+}]_c$ was unaffected (Fig. 4*F*). These results are in agreement with recordings of β-cell membrane potential under similar conditions (33,34).

The impact on insulin secretion was more spectacular. The peak and mean first-phase insulin secretion were inhibited when the prestimulatory glucose concentration was 8.5 instead of 3 mmol/l (Fig. 3*A*, *C*, and *E* and Fig. 4*A* and *C*), whereas the mean second phase was similar (Fig. 4*E*). However, the time course of this second phase was no longer flat but ascending, at least with 16.7 and 30 mmol/l glucose (Fig. 3*C* and *E*).

To assess in greater detail the impact of the prestimulatory glucose concentration, experiments were also started with 5.5 and 11.1 mmol/l glucose before stimulation with 16.7 mmol/l glucose. The pattern of insulin secretion and $[Ca^{2+}]_c$ responses are shown in Fig. 3*G* and *H*. First-phase $[Ca^{2+}]_c$ and insulin secretion occurred more rapidly after prestimulation in 5.5 than in 3 mmol/l glucose, but their amplitudes were not significantly affected. The second-phase insulin secretion, however, was ascending (Fig. 3*G*), which indicated that the change in the second phase pattern was not necessarily linked to a decrease in

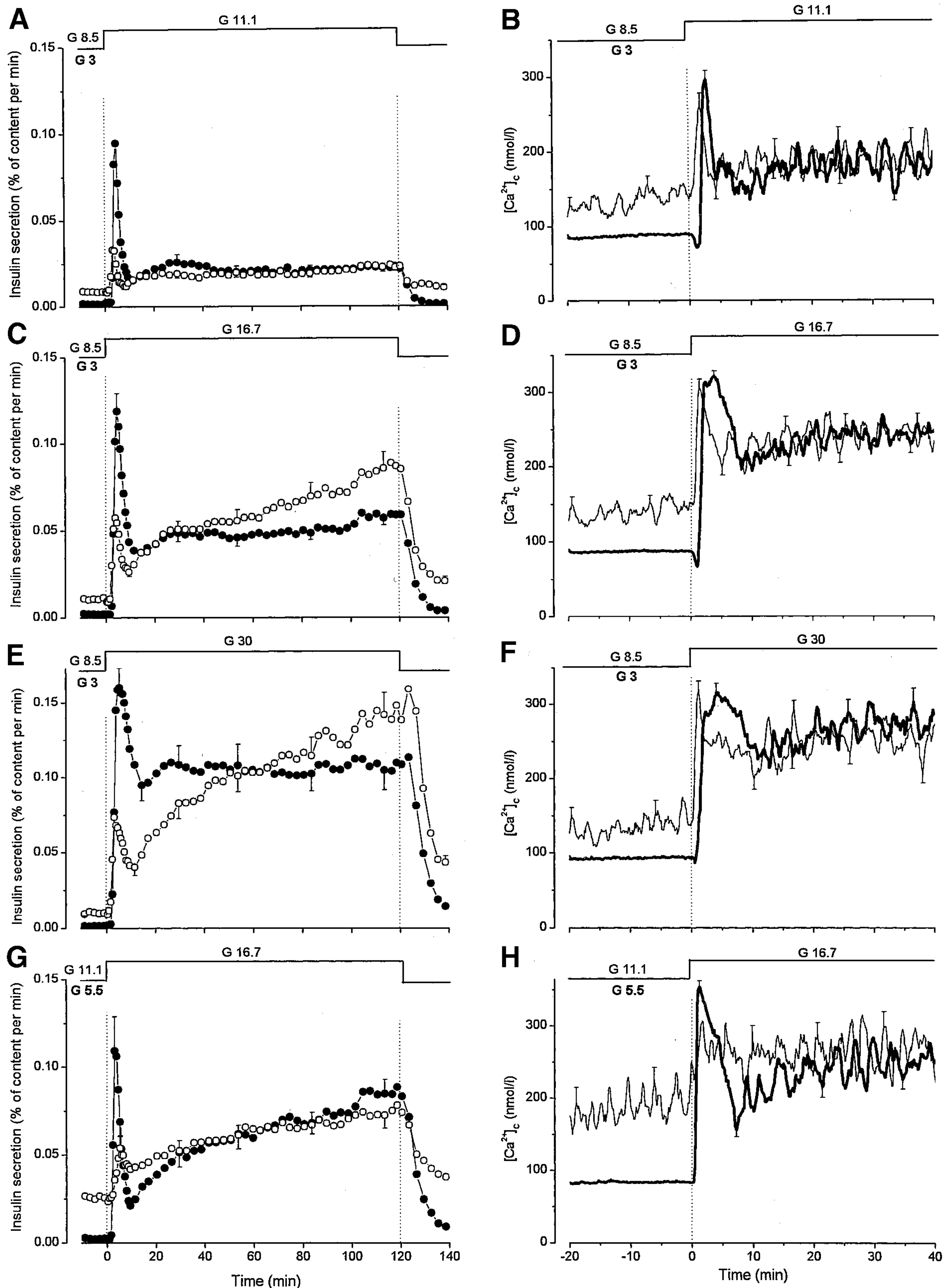

FIG. 3. Biphasic changes in insulin secretion (*A*, *C*, *E*, and *G*) and $[Ca^{2+}]_c$ (*B*, *D*, *F*, and *H*) in mouse islets stimulated by an increase in the glucose concentration of the perifusion medium. *A–F*: As shown on top of each panel, the experiments started with 3 (● and thick lines, G 3) or 8.5 (○ and thin lines, G 8.5) mmol/l glucose. The glucose concentration was then raised to 11.1, 16.7, or 30 mmol/l, as indicated. *G* and *H*: The experiments started with 5.5 (● and thick line, G 5.5) or 11.1 (○ and thin line, G 11.1) mmol/l glucose. The glucose concentration was then raised to 16.7 mmol/l. Data are means ± SE for 7–20 experiments of insulin secretion and 25–72 islets from 5 to 12 preparations for $[Ca^{2+}]_c$.

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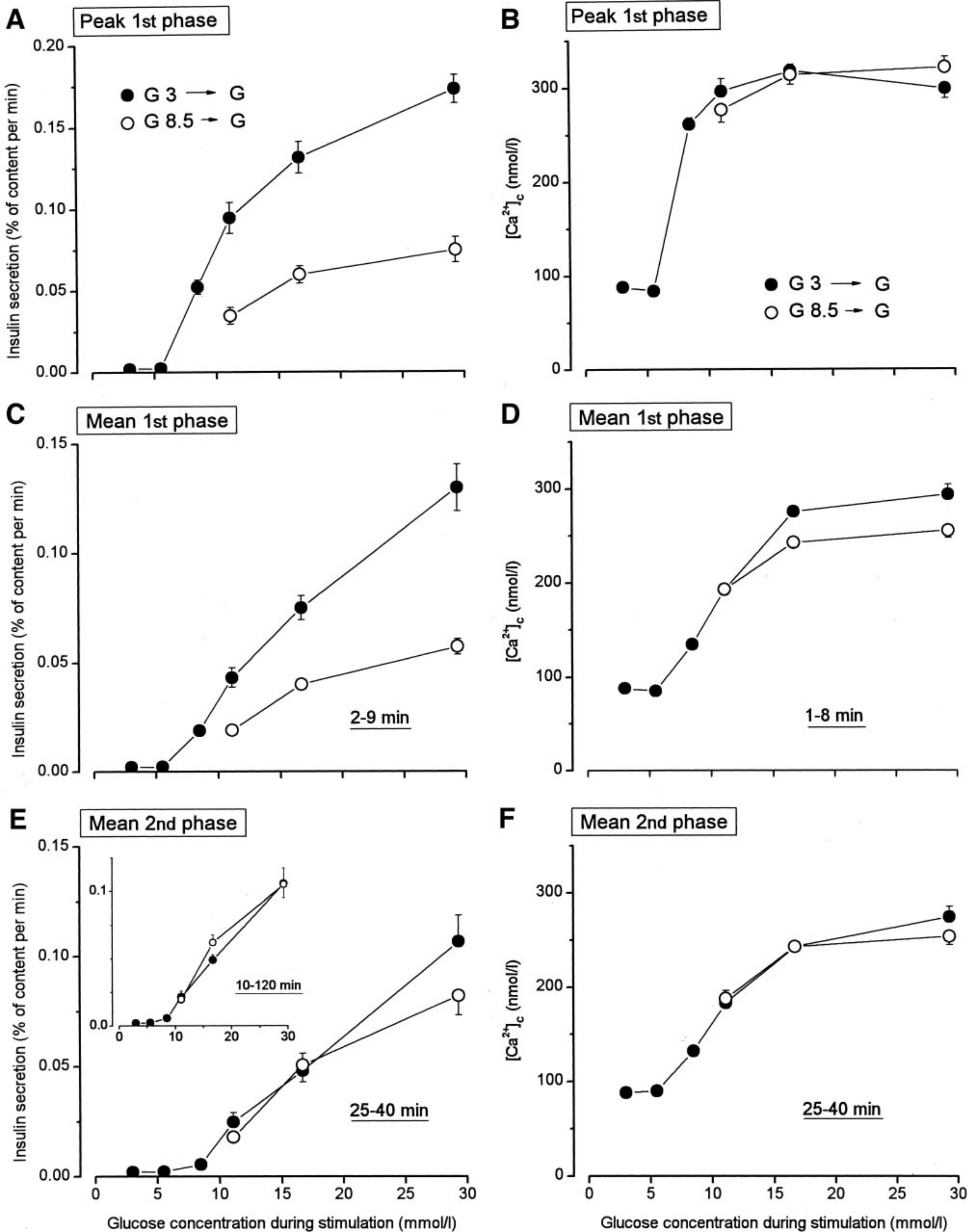

FIG. 4. Concentration dependency of glucose-induced insulin secretion (*A*, *C*, and *E*) and $[Ca^{2+}]_c$ rise (*B*, *D*, and *F*) in perifused mouse islets. Data were computed as described in RESEARCH DESIGN AND METHODS for the experiments shown in Fig. 3*A–F* and similar ones with 5.5 or 8.5 mmol/l glucose. The indicated glucose concentration was tested after a prestimulation period in 3 (●, G 3) or 8.5 (○, G 8.5) mmol/l glucose. Data are means ± SE.

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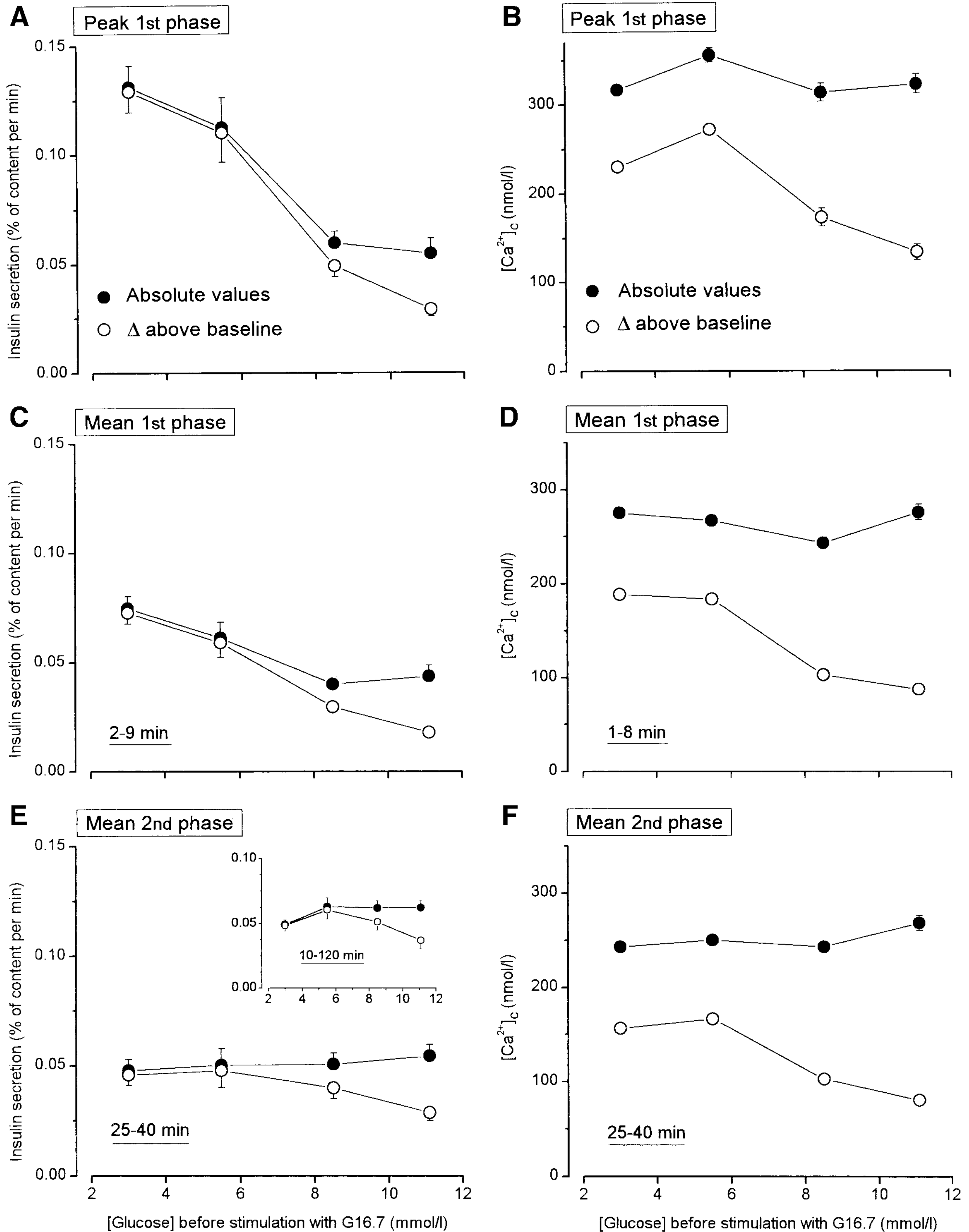

FIG. 5. Influence of the prestimulatory glucose concentration on the biphasic insulin secretion (*A*, *C*, and *E*) and $[Ca^{2+}]_c$ rise (*B*, *D*, and *F*) induced by 16.7 mmol/l glucose (G 16.7). Data were computed as described in RESEARCH DESIGN AND METHODS for the experiments starting with 3, 5.5, 8.5, or 11.1 mmol/l glucose and as shown in panels *C*, *D*, *G*, and *H* of Fig. 3. Both absolute values (●) and differences above baseline (○) are shown. Data are means ± SE.

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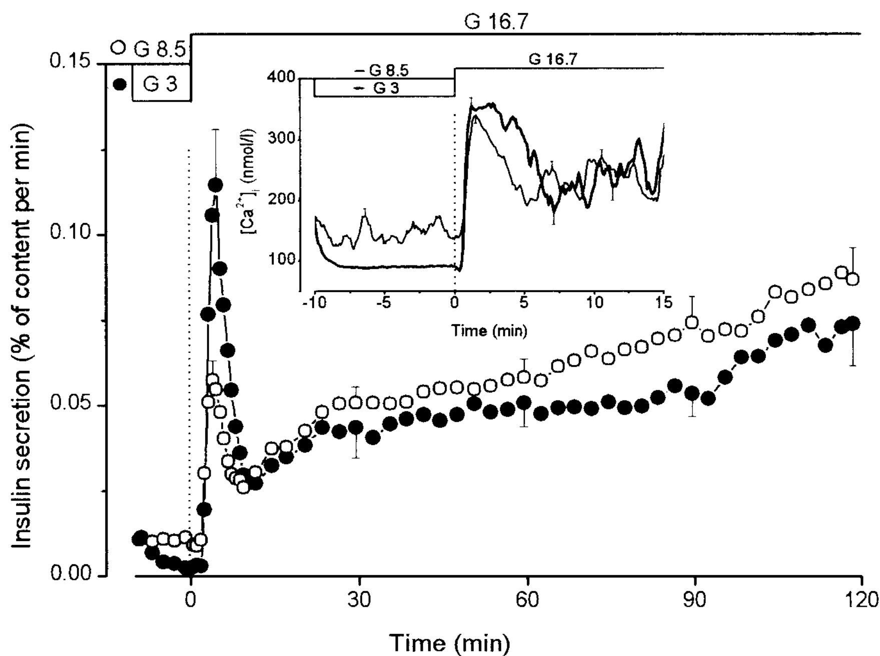

FIG. 6. Influence of a brief lowering of the prestimulatory glucose concentration on the biphasic insulin secretion and $[Ca^{2+}]_c$ rise induced by 16.7 mmol/l glucose (G 16.7) in mouse islets. The experiments started in the presence of 8.5 mmol/l glucose (G 8.5), which remained present until the stimulation with 16.7 mmol/l glucose in one series (○ and thin line). In the other series, the prestimulatory glucose concentration was lowered from 8.5 to 3 mmol/l for 10 min before stimulation with 16.7 mmol/l glucose (● and thick line). Data are means ± SE for six experiments of insulin secretion and 17 islets from three preparations for $[Ca^{2+}]_c$.

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the first phase. When stimulation by 16.7 mmol/l glucose was preceded by an initial period in 11.1 mmol/l glucose, only a small first phase of insulin secretion occurred and the second phase was ascending (Fig. 3*G*).

From these data and those of Fig. 3*C* and *D*, glucose-dependency curves were calculated. During stimulation with 16.7 mmol/l glucose, absolute values of peak and mean first-phase $[Ca^{2+}]_c$ were little influenced and the mean second-phase $[Ca^{2+}]_c$ was unaffected by prestimulatory glucose (Fig. 5*B*, *D*, and *F*). Of course, increments above baseline were smaller ($P < 0.01$) when prestimulatory glucose (8.5 and 11.1 mmol/l) already raised $[Ca^{2+}]_c$. In contrast, the peak and mean first-phase insulin secretion induced by 16.7 mmol/l glucose were decreased when the prestimulatory glucose concentration was raised from 3 to 8.5 or 11.1 mmol/l (Fig. 5*A* and *C*). This decrease affected absolute secretory rates and not only increments above baseline ($P < 0.01$). Unlike the first-phase secretion, the mean second-phase insulin secretion induced by 16.7 mmol/l glucose was unaffected by the prestimulatory glucose, except when calculated as the increment above baseline (Fig. 5*E*). Comparison of the $[Ca^{2+}]_c$ and insulin responses showed that both parameters were similarly unaffected during the second phase but distinctly modified during the first phase: absolute insulin secretion decreased whereas absolute $[Ca^{2+}]_c$ did not.

We eventually tested whether the inhibition of first-phase insulin secretion by an elevation of the prestimulatory glucose concentration was rapidly reversible. Lowering the glucose concentration from 8.5 to 3 mmol/l for 10 min before stimulation with 16.7 mmol/l glucose did not affect the peak first-phase $[Ca^{2+}]_c$ but did increase the mean first-phase $[Ca^{2+}]_c$ because of a longer duration (Fig. 6, *inset*). Simultaneously, both peak and mean first-phase insulin secretion were increased ($P < 0.001$). Neither $[Ca^{2+}]_c$ nor total insulin secretion during the second phase was influenced by the transient decrease in prestimulatory glucose concentration. In particular, the ascending pattern of this second phase of insulin secretion persisted (Fig. 6).

DISCUSSION

Biphasic insulin responses in vivo and in vitro. This study showed that during a hyperglycemic clamp in normal mice, plasma insulin concentrations increased after a biphasic time course, with a gradual rise during the second phase. This in vivo behavior of mice is thus similar to that of humans. In vitro, using the traditional protocol of square-wave increase in glucose from a nonstimulatory to a stimulatory concentration (from 3 to 16.7 mmol/l), we confirmed that perifused mouse islets secrete insulin according to a biphasic time course, with a prominent first phase and a sustained but low and flat second phase. It is not well recognized that the pattern of insulin secretion by perifused human islets is also characterized by a sustained, flat second phase (35–37). The two species thus behave similarly, with an apparent discrepancy between in vivo and in vitro responses. However, imposing hyperglycemia in vivo stimulates β-cells that are also under the influence of other nutrients, hormones, and neurotransmitters, whereas in vitro studies characterize the effects of glucose alone. Moreover, changes in plasma insulin are influenced by insulin extraction rates and do not exclusively reflect changes in insulin secretion. It is noteworthy that the insulin secretion rate, calculated from plasma C-peptide levels by deconvolution, is characterized by a virtually flat second phase, whereas plasma insulin levels are increasing during hyperglycemic clamps (26).

Our in vitro studies further showed that the second phase of insulin secretion by mouse islets is ascending when the protocol of stimulation is close to that in vivo, namely when the stimulation by high glucose (16.7 or 30 mmol/l) is applied from a threshold or mildly stimulatory glucose concentration. This change in pattern cannot be attributed to a decrease in the first phase only because it does not occur during stimulation with 11.1 mmol/l glucose (Fig. 3*A*), persists after the restoration of the first phase response by a brief period in 3 mmol/l glucose (Fig. 6), and is observed after prestimulation with 5.5 mmol/l glucose, a condition that does not decrease the first-phase response (Fig. 3*G*). It also cannot be attributed to any

obvious change in the pattern of the $[Ca^{2+}]_c$ rise, but we acknowledge that the difference in the pattern of second-phase insulin secretion became more obvious when the stimulation by high glucose was extended for longer durations that those compatible with $[Ca^{2+}]_c$ measurements in islets. On the basis of these results, we tentatively suggest that the nature or rate of production of amplifying signals might be influenced by the prestimulatory glucose concentration. It should, however, be noted that this change in pattern had only a minor impact on total insulin secretion over 110 min.

In vivo, the glucose dependency of first-phase insulin response is shifted to the left as compared with the second-phase insulin response. This means that the acute plasma insulin rise better reflects the peak insulin secretion rate than the mean secretion during the first phase. Thus the peak first phase in vitro is clearly more sensitive to glucose than is the second phase, whereas the mean insulin secretion rate (or area under the curve) showed a similar glucose dose dependency for both phases, as in the perfused rat pancreas (38,39). Two differences between in vivo and vitro experiments can be noted: there was no in vivo second phase during stimulation with 11.1 mmol/l glucose, and a maximum response was reached at 16.7 as compared with 30 mmol/l glucose in vitro.

Glucose dependency of biphasic $[Ca^{2+}]_c$ and insulin responses in vitro. Stimulation of mouse islets by a rapid increase in glucose from a nonstimulatory to a stimulatory concentration (e.g., from 3 to 16.7 mmol/l) causes an initial transient decrease in basal $[Ca^{2+}]_c$ followed by a biphasic increase characterized by a prominent first phase and oscillations during the second phase (31,40–42). The large number of parallel experiments performed in the present study highlight the similarities between $[Ca^{2+}]_c$ and insulin secretion changes in glucose-stimulated islets. It is particularly striking that the first phases of $[Ca^{2+}]_c$ rise and insulin secretion similarly increased in duration with the glucose concentration (Fig. 3*A–F*). These observations reinforce the idea that the time course of the triggering signal is an important determinant of the biphasic pattern of insulin secretion (15).

The two phases of the $[Ca^{2+}]_c$ increase result from K_{ATP} channel–dependent depolarization, leading to Ca^{2+} influx through voltage-dependent Ca^{2+} channels (31). Only a few studies have characterized the concentration dependency of the glucose-induced $[Ca^{2+}]_c$ rise in mouse β-cells. A sigmoidal dose-response relation was found in isolated cells (43,44), clusters of islet cells (44,45), and intact islets (42). In those experiments, however, the glucose concentration was usually increased stepwise and for short periods, making comparisons between the first and second phases impossible. Here we showed that the dose-response curve is sigmoidal for both phases of $[Ca^{2+}]_c$ rise, but with several differences. The peak first-phase $[Ca^{2+}]_c$ was already maximal at 11.1 mmol/l glucose and the K_m value was ~7.5 mmol/l glucose, whereas the mean first-phase (computed over 7 min) and second-phase $[Ca^{2+}]_c$ was maximum only at 16.7 mmol/l glucose, with K_m values of ~11 mmol/l glucose.

Because insulin secretion and the $[Ca^{2+}]_c$ were measured over similar periods of time, both variables could be quantitatively compared. The dose-response curve for glucose-induced insulin secretion was consistently shifted to the right compared with that of $[Ca^{2+}]_c$ changes. The increase in β-cell $[Ca^{2+}]_c$ was about maximum at the concentration of glucose causing half-maximum insulin secretion. The contribution of an amplifying pathway (augmentation of exocytosis for a given $[Ca^{2+}]_c$ elevation) to nutrient-induced insulin secretion has previously been established under conditions where $[Ca^{2+}]_c$ was experimentally clamped at an elevated level (19). The present approach did not interfere with glucose-induced $[Ca^{2+}]_c$ changes, but the observed shifts between the $[Ca^{2+}]_c$ and insulin response curves provide a novel, more physiological demonstration that glucose produces signals other than the $[Ca^{2+}]_c$ rise to stimulate insulin secretion. Such a shift was expected for the second phase, during which the amplifying pathway is known to be operative (18,19). Our finding of a similar shift during the peak or mean first phase suggests that the amplifying pathway also contributes to first-phase insulin secretion. However, two caveats should be considered. Classic measurements of first-phase insulin over a fixed period (7 min in our study) could include a contribution of the beginning of the second phase, in particular at the higher glucose concentrations, and the amplification could affect that component only. Peak insulin values should be free of such a contribution, but peak $[Ca^{2+}]_c$ values could be incorrect if the fura-PE3 technique was unable to detect further increases produced by the higher glucose concentrations at the site of exocytosis (17). Measurements of submembrane $[Ca^{2+}]_c$ with a low-affinity indicator could perhaps resolve this issue. However, our control experiments showed that the addition of KCl or arginine on top of the glucose-induced first phase caused a sizeable further rise in the $[Ca^{2+}]_c$ that we would have detected if glucose had produced it. Moreover, our measurements are also compatible with membrane potential recordings showing that it is the period of spike activity (Ca^{2+} action potentials) rather than the size or number of spikes per second that increases with the glucose concentration during the initial minutes of stimulation (42,46). Finally, experiments in which insulin secretion was induced by imposed $[Ca^{2+}]_c$ pulses of controlled amplitude indicate that the amplifying pathway is set in operation rapidly enough to be implicated in the first phase of glucose-induced insulin secretion (47). We acknowledge that amplification has no apparent role in the rapid and short-lived (lasting a few seconds) release of insulin measured by membrane capacitance changes in single β-cells (17,48). We propose, however, that the amplifying pathway together with the indispensable and primary triggering action of Ca^{2+} does contribute to the normal (lasting a few minutes) first-phase insulin secretion that glucose produces in intact islets.

Influence of prestimulatory glucose on the first phase. Our in vitro study also showed that the glucose concentration preceding the square-wave stimulation by high glucose has a strong influence on first-phase insulin secretion. When prestimulatory glucose was increased above 5.5 mmol/l, the incremental and absolute peak and mean first phase induced by 16.7 mmol/l glucose decreased. This inhibitory effect of elevated prestimulatory glucose is not simply explained by smaller glucose increments during stimulation. Thus the mean first phase induced by a 8.5 → 30 mmol/l glucose stimulation was smaller than that induced by a 3 → 16.7 mmol/l glucose jump, although the glucose increment was larger. The inhibition of first-phase insulin secretion also does not seem to be attributable to a smaller triggering signal. The peak $[Ca^{2+}]_c$ was neither decreased by a rise in prestimulatory glucose nor increased after a 10-min period in low glucose (3 mmol/l), which restored a normal first-phase

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insulin secretion. We noted, however, that high prestimulatory glucose often led to a shortening of the first-phase $[Ca^{2+}]_c$. It is also possible that not only absolute $[Ca^{2+}]_c$ but also the amplitude of the change is critical to triggering the rapid secretory response (15).

As an alternative explanation, could our observations be attributed to changes in the size of the pool of readily releasable granules? This pool is thought to comprise ~100 of the ~11,000 granules contained in a mouse β-cell (48,49). Assuming that insulin secretion was stable during the 40 min of prestimulation (measurements were made only during the last 10 min), we calculated that each β-cell released ~35 more granules when the islets were perifused with 8.5 compared with 3 mmol/l glucose. When the experiments started with 8.5 instead of 3 mmol/l, first-phase insulin secretion (over 7 min) induced by 11.1, 16.7, and 30 mmol/l glucose corresponded to ~15 vs. 33, ~30 vs. 58, and ~45 vs. 100 granules per β-cell, respectively. An insufficient number of granules in the readily releasable pool, therefore, does not seem to explain the first-phase decrease induced by 11.1 or 16.7 mmol/l glucose after prestimulation in 8.5 mmol/l glucose. This conclusion is borne out by the fact that the difference in first-phase amplitude corresponds to a similar relative (not absolute) inhibition of 45–52% at the three glucose concentrations. It thus seems unlikely that the phenomenon could be explained only by changes in pool size. However, we wish to emphasize that our calculations and similar ones by others (17,18) assume that all β-cells of the islet secrete insulin at the same rate. Although electrical coupling reduces the heterogeneity and synchronizes $[Ca^{2+}]_c$ changes (50), there is no proof that the secretory response is similar.

Pathophysiological implications. This paradoxical inhibition of first-phase glucose-induced insulin secretion by an elevation of prestimulatory glucose has an in vivo counterpart in humans. Two studies using hyperglycemic clamps in normal subjects, the most recent one calculating the insulin secretion rate by deconvolution, have shown that a moderate elevation of blood glucose reduces first-phase insulin secretion in response to glucose increments (51,52). A progressive decrease in acute insulin response to glucose has also been reported with a mild increase in fasting plasma glucose in normal individuals (12,13). We acknowledge, however, that there are reports showing that the acute insulin response to intravenous glucose is not affected by several hours of imposed hyperglycemia (53). In any event, in agreement with clinical studies (9,12,13), we would like to suggest that minor increases in initial blood glucose might secondarily depress acute insulin responses to high glucose.

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