Evidence That Glucose Can Control Insulin Release Independently from Its Action on ATP-sensitive K^+ Channels in Mouse B Cells

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Abstract

Glucose stimulation of insulin release involves closure of ATP-sensitive K^+ channels, depolarization, and Ca^{2+} influx in B cells. Mouse islets were used to investigate whether glucose can still regulate insulin release when it cannot control ATP-sensitive K^+ channels. Opening of these channels by diazoxide (100–250 μmol/liter) blocked the effects of glucose on B cell membrane potential (intracellular microelectrodes), free cytosolic Ca^{2+} (fura-2 method), and insulin release, but it did not prevent those of high K (30 mmol/liter). K-induced insulin release in the presence of diazoxide was, however, dose dependently increased by glucose, which was already effective at concentrations (2–6 mmol/liter) that are subthreshold under normal conditions (low K and no diazoxide). This effect was not accompanied by detectable changes in B cell membrane potential. Measurements of ^{45}Ca fluxes and cytosolic Ca^{2+} indicated that glucose slightly increased Ca^{2+} influx during the first minutes of depolarization by K, but not in the steady state when its effect on insulin release was the largest. In conclusion, there exists a mechanism by which glucose can control insulin release independently from changes in K^+-ATP channel activity, in membrane potential, and in cytosolic Ca^{2+}. This mechanism may serve to amplify the secretory response to the triggering signal (closure of K^+-ATP channels – depolarization – Ca^{2+} influx) induced by glucose. (*J. Clin. Invest.* 1992. 89:1288–1295.) Key words: diazoxide • pancreatic islets • stimulus-secretion coupling • membrane potential • calcium

Introduction

Insulin secretion is subject to control by nutrients and by hormonal, neural, and pharmacological factors. Among all of these agents, glucose is by far the most important regulator of pancreatic B cell function. The mechanisms by which glucose exerts its effects are complex. However, there is general consensus that a metabolic control of ionic events in B cells is a pivotal step in stimulus-secretion coupling (1–7).

Fig. 1 *A* schematizes the basic mechanisms by which glucose triggers insulin release. The entry of glucose in B cells is followed by an acceleration of metabolism that generates one or several signals that close ATP-sensitive K^+ channels (K^+-ATP channels)[1] in the plasma membrane. The resulting decrease in K^+ conductance leads to depolarization of the membrane with subsequent opening of voltage-dependent Ca^{2+} channels. Ca^{2+} influx through these channels then increases, leading to a rise in cytoplasmic free Ca^{2+}, which eventually activates an effector system responsible for exocytosis of insulin-containing granules. Closure of K^+-ATP channels not only underlies the depolarization brought about by low concentrations of glucose, it also subserves the lengthening of the rhythmic oscillations of membrane potential (responsible for the intermittent influx of Ca^{2+}), which occurs when the concentration of glucose is increased within a physiological range (8–10). As of today, K^+-ATP channels are the only unambiguously identified target on which changes in B cell metabolism act to control insulin release.

The present study was an attempt to determine whether glucose is still able to control insulin release when it cannot exert its effects on K^+-ATP channels. The effects of glucose on K^+-ATP channels were prevented by diazoxide, a drug that directly and selectively opens these channels (11–14) without interfering with B cell metabolism (15, 16). However, since opening of K^+-ATP channels by diazoxide holds the membrane potential at the resting level (17), voltage-dependent Ca^{2+} channels are not activated, Ca^{2+} influx does not occur, and insulin release is not stimulated (Fig. 1 *B*). The membrane was therefore depolarized by increasing the concentration of extracellular K from 4.8 to 30 mM. Under these conditions Ca^{2+} influx and insulin release are again stimulated (Fig. 1 *C*). It thus becomes possible to test the effects of glucose on insulin release independently of changes in K^+-ATP channel activity.

Methods

Solutions. The medium used was a bicarbonate-buffered solution that contained the following (mM): 120 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, and 24 $NaHCO_3$. It was gassed with O_2/CO_2 (94:6) to maintain pH 7.4 and was supplemented with BSA (1 mg/ml). Ca^{2+}-free solutions were prepared by replacing $CaCl_2$ with $MgCl_2$ and adding 100 μM of EGTA.

Measurements of insulin release and ^{45}Ca efflux from islet cells. All experiments were performed with islets isolated by collagenase digestion of the pancreas of fed female NMRI mice (25–30 g), killed by decapitation.

In the first type of experiments, the islets were preincubated for 60 min in a medium containing 15 mM glucose before being distributed

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1. *Abbreviation used in this paper:* K^+-ATP channels, ATP-sensitive K^+ channels.

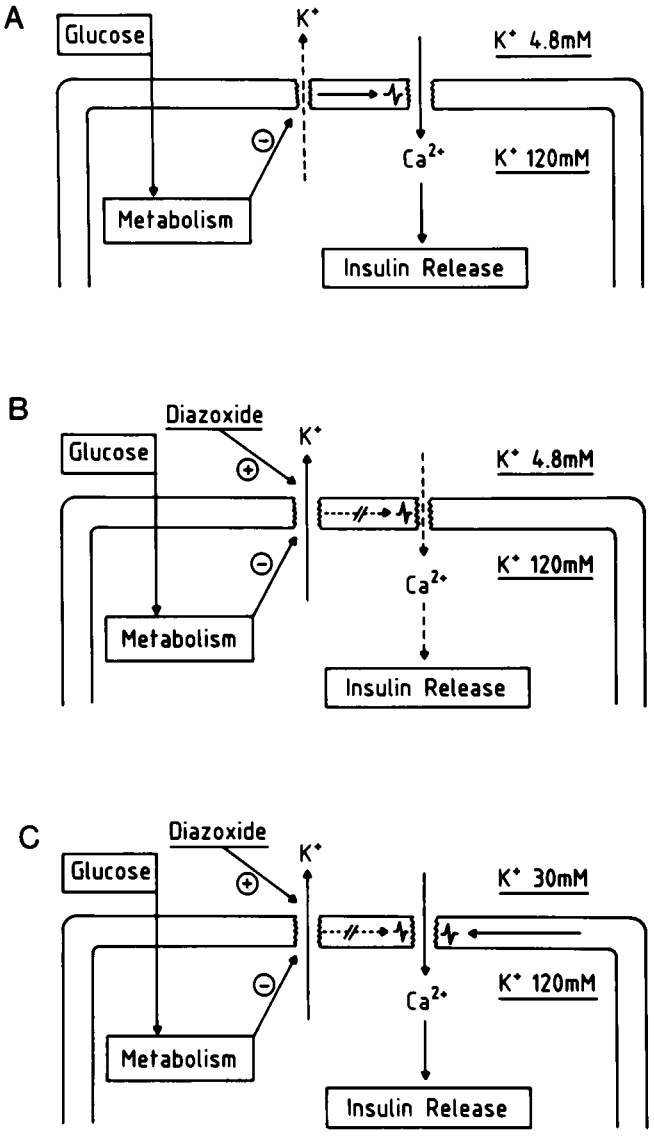

Figure 1. Schematic representation of the mechanisms by which glucose controls insulin release through changes in B cell membrane potential and of our experimental approach to demonstrate the existence of an additional mechanism.

into batches of three. Each batch of islets was then incubated for 60 min in 1 ml of medium containing appropriate concentrations of glucose and test substances. A portion of the medium was withdrawn at the end of the incubation and appropriately diluted for insulin assay. Insulin was measured by a double-antibody radioimmunoassay with rat insulin as the standard (Novo Research Institute, Bagsvaerd, Denmark).

In another type of experiments, the isolated islets were first loaded with ^{45}Ca during 90 min of incubation in 0.25 ml of medium containing 15 mM glucose and supplemented with $^{45}CaCl_2$ (15 MBq/ml; sp act. 6 GBq/mmol). The islets were then washed, placed in batches of 30 in parallel perifusion chambers, and perifused at a flow rate of 1.25 ml/min (18). The radioactivity lost by the islets was measured in effluent fractions collected at 2-min intervals, and the fractional efflux rate was calculated for each period. A portion of each effluent fraction was also taken for insulin assay.

Recordings of B cell membrane potential. A piece of mouse pancreas was fixed in a perifusion chamber and a few islets were partially microdissected by hand. The membrane potential of a single cell within the islet was continuously measured with a high resistance electrode (19). B cells were identified by the typical electrical activity that they display in the presence of 10–15 mM glucose. The temperature was 37°C, and the perifusion medium was similar to that described above except for the absence of albumin.

Measurements of intracellular Ca^{2+} in islet cells. Isolated islets were cultured for 1–4 d in RPMI 1640 medium containing 10% heat-inactivated FCS, 100 IU/ml of penicillin and 100 μg/ml of streptomycin. The concentration of glucose was 10 mM.

Islets were loaded with fura-2 during 35 min of incubation at 37°C in 2 ml of medium containing 10 mM glucose and 0.9 μM fura-2 acetoxymethylester (Molecular Probes, Inc., Eugene, OR; added from a 1 mM stock solution in DMSO). The loaded islet was then transferred into a temperature-controlled perifusion chamber (Joyce, Loebl and Co. Ltd., Gateshead, U.K.) with a bottom made of a glass coverslip. The islet was held in place by gentle suction with a glass micropipette and perifused at a flow rate of 1.3 ml/min. The perifusion solutions were kept at 37°C in a water bath, and the temperature controller was set to ensure a constant temperature of 37°C (±0.3°C) close to the islet, as monitored by a thermistor placed near the tissue.

The perifusion chamber was mounted on the stage of an inverted microscope (Nikon Diaphot) used in the epifluorescence mode with a 20× objective. Fura-2 was successively excited at 340 and 380 nm by means of two narrow band-pass filters mounted on a computer-controlled motorized filter wheel placed in front of a 75 W xenon lamp. A dichroic mirror centered at 430 nm reflected the ultraviolet (UV) light to the perifusion chamber and transmitted the emitted fluorescence, which was further filtered through a 510-nm filter. Fluorescent images were obtained with a CCD video camera (Photonic Science Ltd., Tunbridge Wells, U.K.) at a resolution of 256 × 256 pixels. They were then digitized into 256 grey levels and analyzed with the system Magical of Joyce, Loebl and Co. Ltd. To improve the signal/noise ratio, eight consecutive 40-ms frames were averaged at each wavelength before calculating the ratio. The time interval between successive series of 340 to 380 images was 12 s. During these intervals, the excitation light was stopped to avoid photobleaching of fura-2. The concentration of cytoplasmic Ca^{2+} was calculated by comparing the ratio of the fluorescence at each pixel with an in vitro calibration curve based on the equation of Grynkiewicz et al. (20) as previously described (21). The K_D for Ca^{2+}-fura-2-complex employed was 224 nM. The mean Ca^{2+} concentration in the islet was then calculated by averaging the Ca^{2+} concentration at all pixels of the islet.

Materials. Diazoxide was obtained from Schering Corp. (Bloomfield, NJ), mannoheptulose and 3-*O*-methylglucose were obtained from Sigma Chemical Co., (St. Louis, MO), α-ketoisocaproate was obtained from Aldrich Chemie (Steinheim, Germany), and all other reagents were obtained from E. Merck (Darmstadt, Germany). $^{45}CaCl_2$ was obtained from the Radiochemical Center (Amersham Corp., Bucks., U.K.).

Presentation of results. Electrophysiological experiments are illustrated by recordings that are representative of the indicated number of experiments. Otherwise, results are presented as means (±SEM) for the indicated number of experiments (different animals or islet preparations) or batches of islets. The statistical significance of differences between means was assessed by Student's *t* test for unpaired data or by analysis of variance followed by a test of Newman-Keuls when more than two groups were compared. Differences were considered significant at $P < 0.05$.

Results

Insulin release by incubated islets. Basal insulin release by islets incubated in a medium containing 4.8 mM K and no glucose amounted to 0.43±0.3 ng/islet per h (n = 20). It was not affected by 100–250 μM diazoxide (Fig. 2). 30 mM of glucose stimulated insulin release 26-fold (11.0±1.5 ng/islet per h); this effect was abolished by diazoxide. On the other hand, the 16-

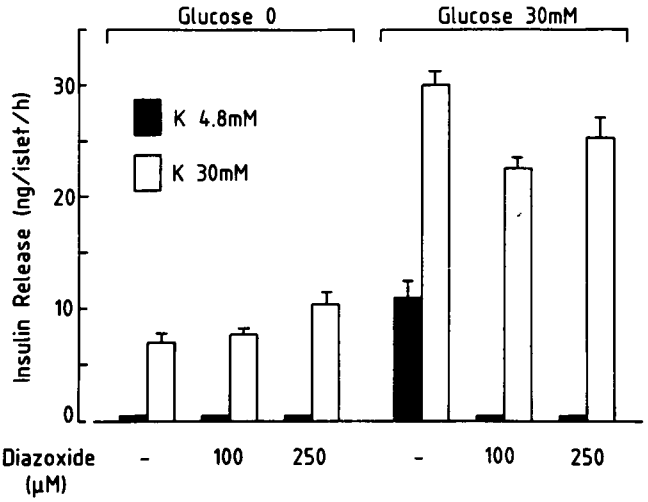

Figure 2. Effects of diazoxide on glucose- and K-induced insulin release by incubated mouse islets. Batches of three islets were incubated for 1 h in 1 ml of medium containing the indicated concentrations of glucose and K. The medium was supplemented with 100–250 μM diazoxide or no diazoxide was added. Values are mean±SEM for 20 batches of islets.

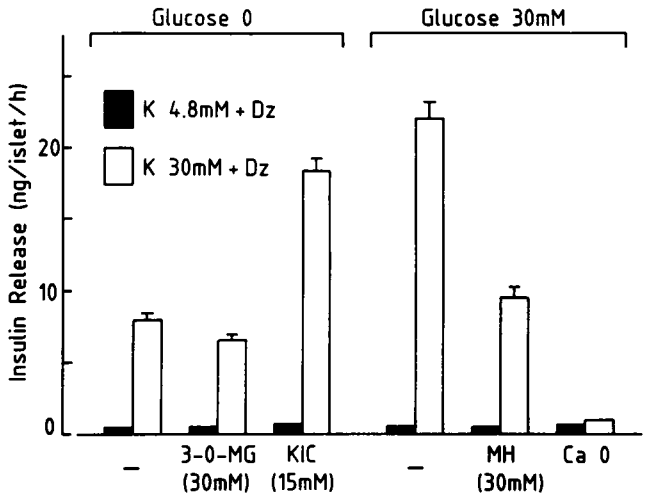

Figure 3. Effects of various experimental conditions on K-induced insulin release by incubated mouse islets. The medium always contained 100 μM diazoxide (*Dz*) and either no or 30 mM glucose as indicated. The experimental conditions tested were 3-*O*-methylglucose (*3-O-MG*), α-ketoisocaproate (*KIC*), mannoheptulose (*MH*) at the indicated concentrations, and omission of extracellular Ca (*Ca* 0). Values are mean±SEM for 22 batches of islets.

fold increase in insulin release brought about by 30 mM K in the absence of glucose was not inhibited by diazoxide. High K also markedly increased insulin release in the presence of 30 mM glucose (Fig. 2). Although this response to high K was slightly attenuated by both concentrations of diazoxide ($P < 0.01$), the net increase was ~ 2.5-fold larger than when the medium contained diazoxide but no glucose ($P < 0.01$; analysis of variance and test of Newman-Keuls). Glucose is thus able to increase insulin release in response to K, even when its genuine effect on release is abolished by diazoxide.

The experiments shown in Fig. 3 were all performed in the presence of 100 μM diazoxide. It can be seen that 3-*O*-methylglucose, a nonmetabolized analogue of glucose (22), did not increase the insulin response to 30 mM K, whereas α-ketoisocaproate, which is well metabolized (23), increased it 2.3-fold ($P < 0.01$). Fig. 3 also shows that the effect of glucose was strongly inhibited, though not abolished, by mannoheptulose, an inhibitor of glucose metabolism (15). Finally, omission of extracellular Ca totally prevented the stimulation of insulin release by high K in 30 mM glucose. The amplification of K-induced insulin release by glucose thus requires metabolism of the sugar in B cells and depends upon the availability of extracellular Ca.

Fig. 4 shows the sigmoidal relationship between the concentration of glucose in the incubation medium and insulin release under control conditions. It also shows that the effect of all concentrations of glucose was abolished by 100 μM diazoxide. In 30 mM K, however, glucose again produced a dose-dependent increase in insulin release, in spite of the presence of diazoxide. This dose–response curve displayed two components. A first increase occurred between 0 and 3 mM glucose ($P < 0.05$), and a second increase occurred at > 6 mM. The initial increase was characterized in greater detail in another series of experiments (Fig. 4, *inset*). A significant amplification of K-induced insulin release was observed at 2 mM glucose ($P < 0.01$). The effect of glucose then plateaued between 3 and 6 mM. Half-maximal stimulation of insulin release was produced by 15.5 mM glucose under control conditions (4.8 mM K) and by 11.8 mM glucose in the presence of 30 mM K and diazoxide.

^{45}Ca efflux and insulin release from perifused islets. These experiments were performed in the presence of diazoxide throughout. Increasing the concentration of K from 4.8 to 30 mM in a medium containing 3 mM glucose and 100 μM diazoxide induced a rapid, marked, and monophasic acceleration of ^{45}Ca efflux from perifused islets, and simultaneously induced a biphasic release of insulin (Fig. 5). The second phase was, however, not sustained. When the concentration of glucose was increased from 3 to 20 mM in the presence of 30 mM K, the rate of ^{45}Ca efflux transiently decreased before increasing marginally above control values (Fig. 5 *A*). Although the difference was small, the steady state rate of ^{45}Ca efflux was consistently higher in 20 than in 3 mM glucose ($P < 0.05$, paired *t* test). The change in glucose concentration also caused a slightly delayed, marked, and reversible increase in insulin release (Fig. 5 *B*).

Increasing the concentration of K to 30 mM in a medium containing 250 μM diazoxide triggered a faster and larger ($P < 0.001$) acceleration of ^{45}Ca efflux from the islets perifused with 20 rather than 3 mM glucose (Fig. 6 *B*). In the steady state, however, the rate of ^{45}Ca efflux was similar; although mean values were slightly higher in islets perifused with 20 rather than 3 mM glucose, this was not observed in all paired experiments. In contrast, insulin release was much larger in 20 rather than 3 mM glucose during both the early and late phases of the response to 30 mM K (Fig. 6 *B*). When these experiments were performed in a Ca^{2+}-free medium, neither ^{45}Ca efflux nor insulin release was stimulated by high K (not shown).

Membrane potential of B cells. In the presence of 15 mM glucose, B cells exhibited a rhythmic electrical activity consisting of slow waves of the membrane potential with Ca^{2+} spikes superimposed on the plateau (Fig. 7, *A* and *B*). Increasing the concentration of K to 30 mM persistently depolarized the membrane at a potential that was 13±1 mV less negative than

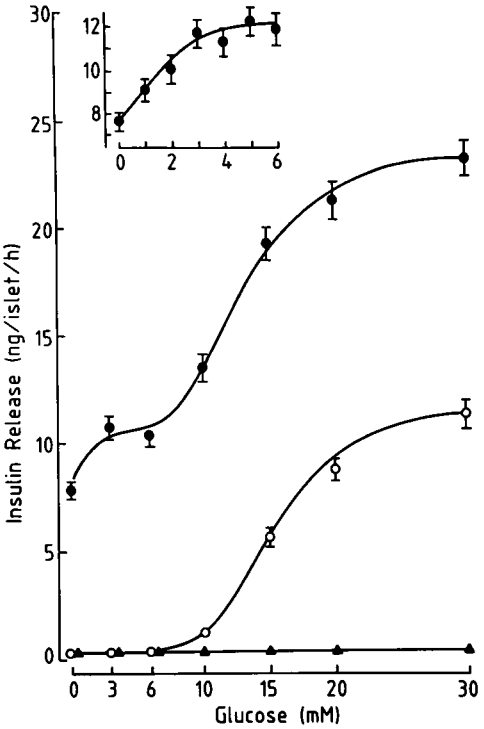

Figure 4. Concentration dependency of glucose-induced insulin release by mouse islets incubated: (o —— o) in a control medium containing 4.8 mM K, (▲ —— ▲) in the presence of 100 μM diazoxide and 4.8 mM K, (● —— ●) in the presence of 100 μM diazoxide and 30 mM K. Values are mean±SEM (when larger than the symbol) for 40, 13, and 27 batches of islets, respectively. (*Inset*) Effects of low glucose concentrations on insulin release by mouse islets incubated in the presence of 100 μM diazoxide and 30 mM K. Values are mean±SEM for 20 batches of islets.

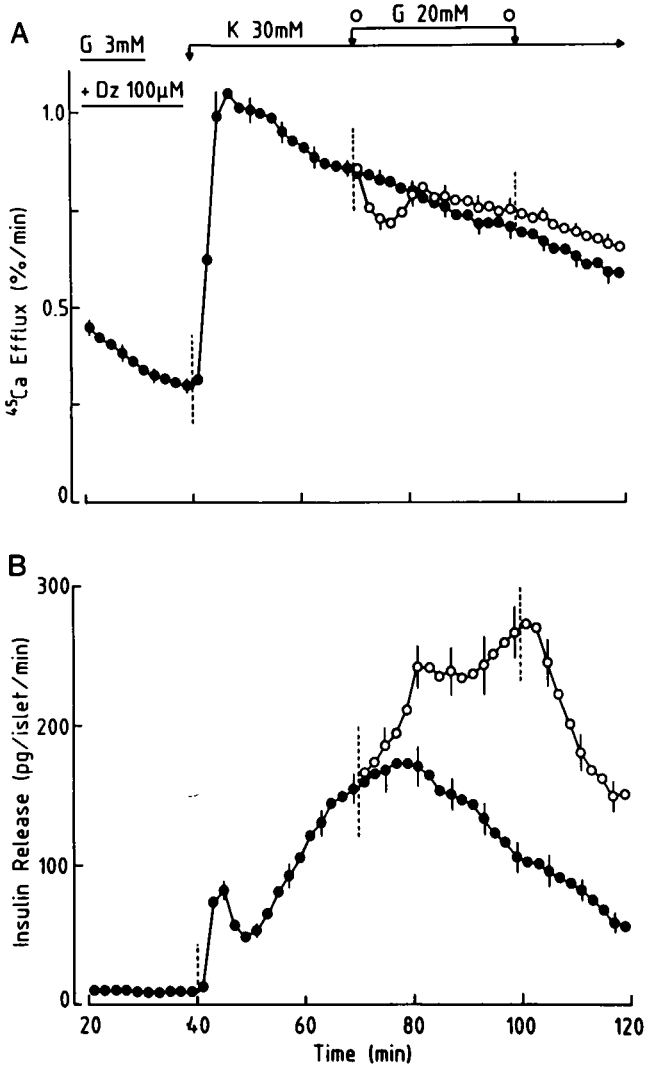

Figure 5. Effects of glucose on the stimulation of ^{45}Ca efflux and insulin release by 30 mM K in mouse islets perifused with a medium containing 100 μM diazoxide (*Dz*) throughout. The experiments were started with a medium containing 3 mM glucose (*G*) and 4.8 mM K. The concentration of K was then increased to 30 mM from 40 min. In one series (o —— o), the concentration of glucose was increased to 20 mM between 70 and 100 min. Values are mean±SEM for six parallel experiments.

the plateau potential. It also induced continuous spike activity, but the amplitude of the spikes decreased with time. After a few minutes, there remained only small fluctuations of the membrane potential, which probably reflect Ca^{2+} channel activity (24). Addition of 100 μM diazoxide repolarized the membrane by 7±1 mV and suppressed the small fluctuations of the membrane potential (Fig. 7 *A*).

When diazoxide was added first to the medium containing 4.8 mM K, glucose-induced electrical activity was abolished and the membrane repolarized to the resting potential of the cell (−69±2 mV) (Fig. 7 *B*). The subsequent increase in K concentration to 30 mM was followed by a rapid depolarization by 40±2 mV, but no electrical activity occurred.

High K similarly depolarized the membrane of B cells perifused with a glucose-free medium containing either 100 μM diazoxide or no diazoxide (Fig. 7, *C* and *D*). However, the subsequent addition of 20 mM glucose had no effect in the presence of diazoxide (Fig. 7 *D*), but it caused a further depolarization by 5.2±0.5 mV and induced appearance of small fluctuations of the membrane potential in the absence of diazoxide (Fig. 7 *C*). Similar results were obtained whether the experiments started in the absence of glucose or in the presence of 3 mM glucose.

Cytoplasmic Ca^{2+} in islet cells. The concentration of cytoplasmic Ca^{2+} in B cells was estimated in whole islets loaded with the Ca indicator, fura-2. A first series of control experiments were made to assess that the recorded signal is representative of the changes in Ca_i^{2+} occurring in B cells. In 3 mM glucose, Ca_i^{2+} averaged 80±5 nM (n = 6). Increasing the concentration of glucose to 20 mM in the perifusion medium caused an initial transient decrease in Ca_i^{2+} (7±2 nM; $P < 0.005$, paired t test) that was followed by a large biphasic rise to a plateau at 194±8 nM. Subsequent addition of 100 μM diazoxide lowered the concentration of Ca_i^{2+} to 93±7 nM in spite of the presence of high glucose. In other experiments, addition of 100 μM tolbutamide to a medium containing 3 mM glucose increased Ca_i^{2+} from 70±6 to 183±10 nM (n = 10). These changes are similar to those recorded in B cell suspensions (25–27) or in single B cells (21). It should also be noted that K_{ATP} channels, the target of diazoxide and tolbutamide (11), are present in B but not in A cells (28).

Fig. 8 *A* shows the mean increase in cytoplasmic Ca^{2+} that occurred in islet cells upon stimulation by 30 mM K in a me-

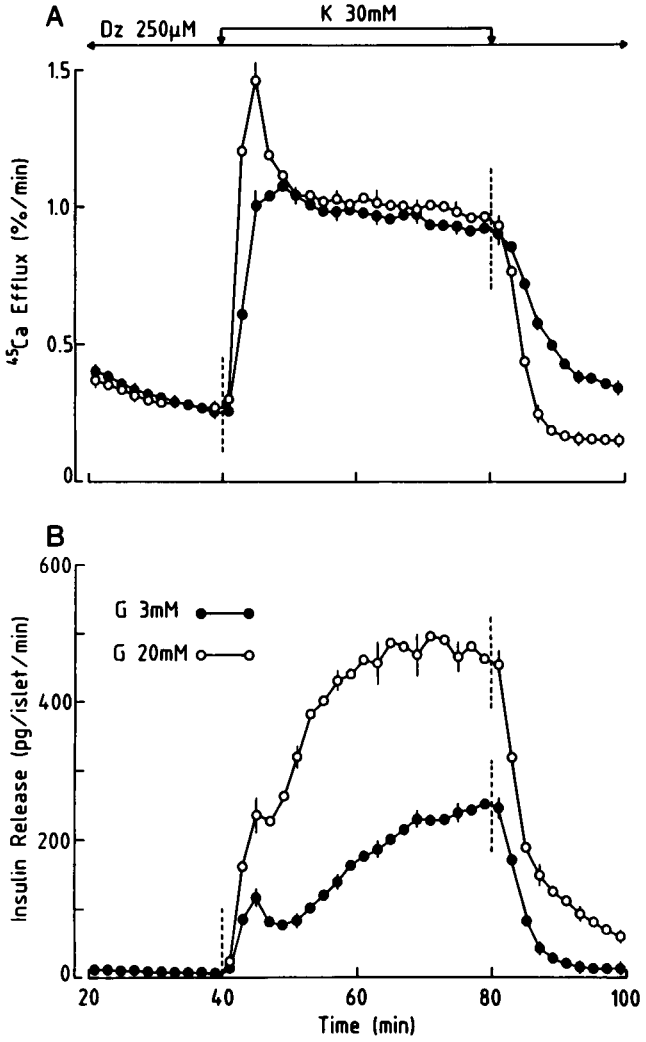

Figure 6. Effects of glucose on the stimulation of ^{45}Ca efflux and insulin release by 30 mM K in mouse islets perifused with a medium containing 250 μM diazoxide (*Dz*) throughout. The concentration of glucose was 3 mM (●) or 20 mM (○). The experiments were started with a medium containing 4.8 mM K. The concentration of K was then increased to 30 mM between 40 and 80 min. Values are mean±SEM for six parallel experiments.

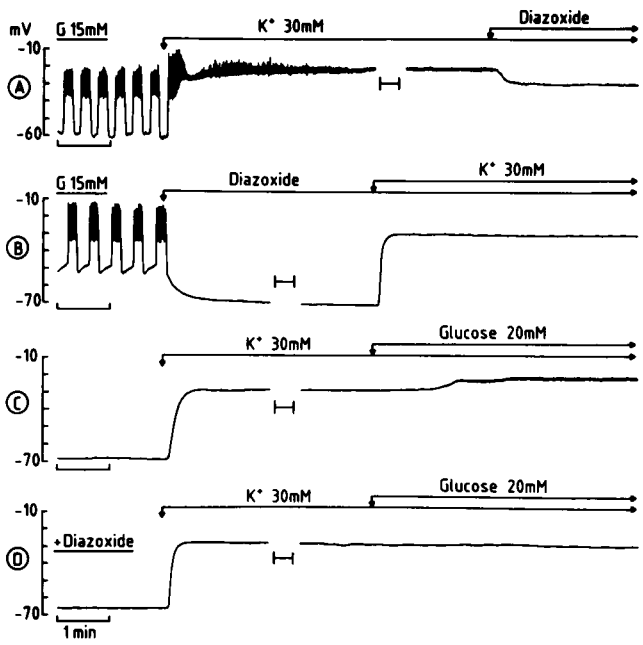

Figure 7. Effects of K and diazoxide on the membrane potential of mouse pancreatic B cells. All changes in the composition of the medium were made at times indicated by the arrows. (*A*) The concentration of K was increased from 4.8 to 30 mM in a medium containing 15 mM glucose (*G*). 10 min later, diazoxide was added at the concentration of 100 μM. (*B*) Diazoxide (100 μM) was first added to a medium containing 15 mM glucose (*G*) and 4.8 mM K. 10 min later, the concentration of K was increased to 30 mM. (*C*) The concentration of K was increased from 4.8 to 30 mM in a glucose-free medium. 10 min later, glucose was added at the concentration of 20 mM. (*D*) Same experiments as in *C*, but in the presence of 100 μM diazoxide throughout. The four recordings were obtained in separate experiments and are representative of results obtained in four different cells (different mice) for *A* and *B* and five different cells for *C* and *D*. In the latter two series, however, three experiments were started in the absence of glucose and the other two were started in the presence of 3 mM glucose and gave similar results.

dium containing 3 mM glucose and 100 μM diazoxide. The concentration of Ca_i^{2+} increased from 82±8 nM in the basal state to a peak of 314±28 nM before declining slightly and stabilizing in plateau. When the concentration of glucose was raised to 20 mM in the presence of 30 mM K, the concentration of cytoplasmic Ca^{2+} rapidly but transiently decreased before returning to values similar to those measured in low glucose. A similar response apparently occurred in all regions of the islets. In the steady state (minute 21 of the experiment), the mean concentrations of cytoplasmic Ca^{2+} were 270±26 (n = 7) and 250±11 nM (n = 8) in 3 and 20 mM glucose, respectively. These values were not significantly different. However, within each experiment, Ca_i^{2+} was lower 10 min after the increase in glucose concentration than just before; the average decrease amounted to 27±4 nM ($P < 0.001$, paired t test). Over the same period of time, there was no change in Ca_i^{2+} in islet cells maintained in a medium with 3 mM glucose (1±3 nM).

The effect of glucose on the initial peak of cytoplasmic Ca^{2+} caused by the increase in K concentration from 4.8 to 30 mM was studied in the presence of 250 μM diazoxide (Fig. 8 *B*). The peak Ca_i^{2+} value was marginally ($0.05 < P < 0.10$) higher in 20 mM glucose (349±14 nM, n = 12) than in 3 mM glucose (315±11 nM, n = 12), but the steady state Ca_i^{2+} concentration was not different.

Discussion

The present study demonstrates that glucose is still able to regulate insulin release when it no longer can control K^+-ATP channel activity and, hence, the membrane potential of B cells. However, this regulation is detectable only when Ca influx is stimulated by high K.

The resting potential of B cells is mainly determined by the high K^+ permeability of the plasma membrane (29). Although this permeability decreases as the glucose concentration is raised (1, 5–7), the membrane potential of B cells remains very sensitive to changes in the equilibrium potential of K^+ (E_K). The shift of E_K to less-negative values when extracellular K was increased caused a sustained depolarization. However, as shown in control experiments, mere depolarization by 30 mM K was not sufficient to clamp the membrane potential and

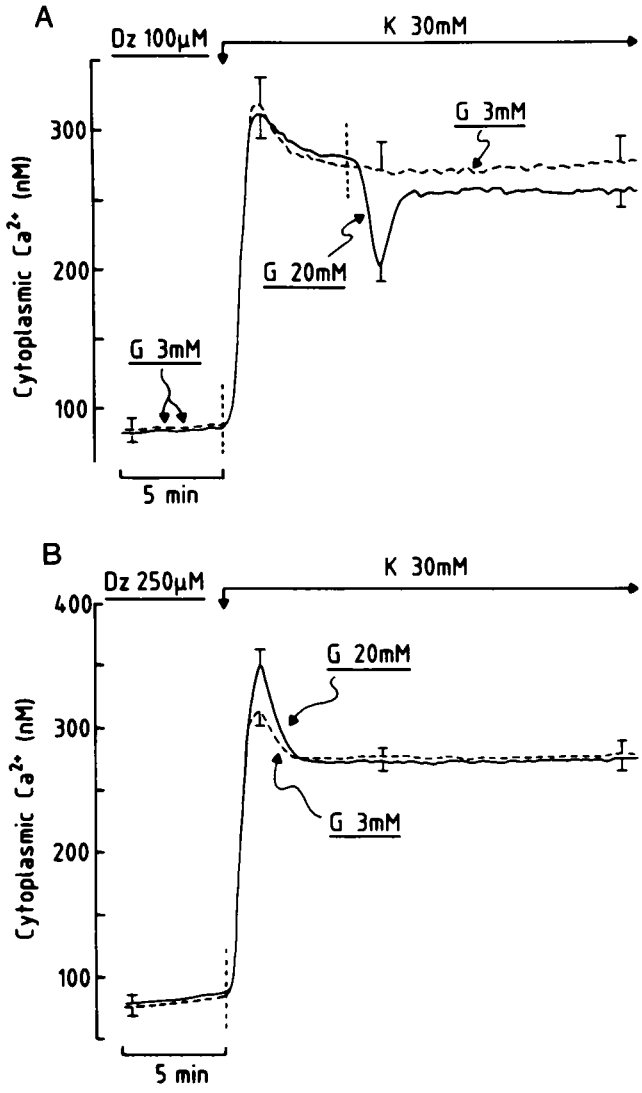

Figure 8. Effects of glucose on the increase in cytoplasmic Ca^{2+} brought about in islet cells by 30 mM K. (*A*) The experiments were made in the presence of 100 μM diazoxide (*Dz*) throughout. They were started in a medium containing 3 mM glucose and 4.8 mM K. The concentration of K was then increased to 30 as indicated by the arrows. In one series (—), the concentration of glucose was raised to 20 mM, 6 min after the increase in K concentration. The traces correspond to the mean response obtained in 7–8 islets. (*B*) The experiments were made in the presence of 250 μM diazoxide (*Dz*) throughout and either 3 mM glucose (- - -) or 20 mM glucose (——). The concentration of K was increased from 4.8 to 30 mM as indicated by the arrows. The traces correspond to the mean response obtained in 12 islets.

make it insensitive to glucose. The use of diazoxide was indispensable to prevent the effects of glucose on the membrane potential.

Although 100 μM diazoxide repolarized the B cell membrane to the resting potential (8) and abolished insulin release (this study) even in the presence of 30 mM glucose, certain experiments were also performed using 250 μM diazoxide. As the effectiveness of diazoxide on K^+-ATP channels decreases at high concentrations of intracellular ATP (12–14), we did not wish to incur the slightest risk of using too low a concentration of the drug; it is also known that ~ 10% of diazoxide is bound in solutions containing 1 mg/ml albumin (30). Since the results obtained with both concentrations of diazoxide were superimposable, one can safely conclude that none of the effects produced by glucose is attributable to any significant closure of K^+-ATP channels under our experimental conditions.

As expected from previous studies, opening K^+-ATP channels by diazoxide prevented glucose from depolarizing the B cell membrane and inducing a Ca^{2+}-dependent electrical activity (17), from increasing cytosolic Ca_i in B cells (16, 25), and from stimulating insulin release (17, 31). B cells thus apparently became insensitive to glucose. This was certainly not because they did not adequately metabolize the sugar, since high concentrations of diazoxide (400–500 μM) are without effect on glucose oxidation by mouse islets (15, 16). It is also known that diazoxide does not interfere with the stimulation of insulin biosynthesis by glucose at concentrations (435 μM) that abolish the effects on release (32, 33). This can be explained simply by the fact that, in contrast to secretion, insulin biosynthesis is Ca^{2+}-independent (34). The effect of glucose on insulin release in the presence of diazoxide could be disclosed only when Ca^{2+} influx, the triggering signal, was restored by depolarizing the membrane with high K. This effect was clearly dependent on the metabolism of the sugar, as it was not mimicked by the nonmetabolizable 3-*O*-methylglucose, was largely prevented by mannoheptulose, and was reproduced by α-ketoisocaproate.

That high K can induce insulin release in the presence of diazoxide has long been known (35, 36). The key observation of the present study was that glucose increased the secretory response to K in spite of the impossibility for the sugar to close K^+-ATP channels. Most surprisingly, the dose dependency of this effect of glucose was not the same as that measured under control conditions. A significant increase in release was already produced by 2 mM glucose and the half-maximal response was observed at 11.8 mM glucose. These values contrast with the control values of ~ 7 mM for the threshold and 15.5 mM for the half-maximally effective concentrations, respectively. The curve relating the concentration of glucose to insulin release in the presence of high K and diazoxide is reminiscent of the dose–response curves for glucose usage and oxidation by islets studied under normal conditions (37, 38).

Changes in Ca^{2+} handling by B cells are an obvious mechanism by which glucose could augment K-induced insulin release. This possibility was investigated by monitoring ^{45}Ca efflux from preloaded islets and by measuring cytosolic Ca^{2+} in B cells. The immediate response to high K was characterized by a larger acceleration of ^{45}Ca efflux and a slightly higher (marginally significant) rise in Ca_i^{2+} in 20 rather than in 3 mM glucose, which suggests that glucose increased Ca^{2+} influx. Since the change in membrane potential was not different, these observations could be interpreted as evidence supporting the suggestion that nutrient secretagogues may modulate the activity of voltage-dependent Ca^{2+} channels (39, 40). In the steady state, however, the rate of ^{45}Ca efflux and the concentration of cytosolic Ca^{2+} were similar at both glucose concentrations, indicating that the possible effect of glucose on Ca^{2+} influx faded out during sustained depolarization. In the other series of experiments, when the concentration of glucose was increased from 3 to 20 mM in the presence of 30 mM K^+, an initial decrease in cytosolic Ca^{2+} occurred. Since it was accompanied by a de-

crease in the rate of ^{45}Ca efflux, one may conclude that the change in Ca_i^{2+} was not due to accelerated extrusion but to increased sequestration in cellular organelles. This phenomenon is similar to, though larger than, the transient lowering of Ca_i^{2+} that occurs when the concentration of glucose is increased in a normal medium (26) (our control experiments), and which is also ascribed to Ca sequestration. Subsequently, the rate of ^{45}Ca efflux increased to comparable values as those measured in low glucose, whereas the concentration of cytosolic Ca^{2+} tended to remain slightly lower. Therefore, an undisputable, conservative conclusion is that the steady state increase in insulin release brought about by glucose in a medium containing high K and diazoxide is not the consequence of a larger rise in cytosolic Ca^{2+}. The present study was not designed to identify which of numerous coupling factors might underlie the effect of glucose. However, stimulation of protein kinases that increase the efficacy of Ca_i^{2+} on the secretory machinery is a plausible hypothesis.

In conclusion, we have demonstrated the existence of a mechanism by which glucose can control insulin release independently from changes in K^+-ATP channel activity, in membrane potential, and in cytosolic Ca^{2+}. The existence of this mechanism does not detract from the essential role of K^+-ATP channels, which clearly remain the major target for the sugar. If glucose cannot close them to depolarize the membrane and induce Ca^{2+} influx, insulin release is not stimulated. The newly identified mechanism can only amplify the secretory response to the triggering signal induced by glucose. The hypothesis that glucose induces both initiating and potentiating signals in B cells has been raised previously (41). It has also been suggested that the permissive effect of glucose, that is the potentiation by the sugar of insulin release induced by other secretagogues, is partly due to a membrane potential–independent mechanism (42). Finally, the existence of this intracellular mechanism of regulation raises the possibility that B cell dysfunction, as that occurring in noninsulin-dependent diabetes, is not necessarily secondary to defects in K^+-ATP channel control. One might also wonder whether abnormalities of the intracellular mechanism of control do not contribute to primary or secondary failures of treatments with sulfonylureas, which only act on K^+-ATP channels.

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References

1. Henquin, J. C., and H. P. Meissner. 1984. Significance of ionic fluxes and changes in membrane potential for stimulus-secretion coupling in pancreatic B-cells. *Experientia (Basel).* 40:1043–1052.

2. Wollheim, C. B., and T. J. Biden. 1986. Signal transduction in insulin secretion: comparison between fuel stimuli and receptor agonists. *Ann. NY Acad. Sci. USA.* 488:317–333.

3. Prentki, M., and F. M. Matschinsky. 1987. Ca^{2+}, cAMP, and phospholipid-derived messengers in coupling mechanisms of insulin secretion. *Physiol. Rev.* 67:1185–1248.

4. Wolf, B. A., J. R. Colca, J. Turk, J. Florholmen, and M. L. McDaniel. 1988. Regulation of Ca^{2+} homeostasis by islet endoplasmic reticulum and its role in insulin secretion. *Am. J. Physiol.* 254:E121–E136.

5. Cook, D. L., L. S. Satin, M. L. J. Ashford, and C. N. Hales. 1988. ATP-sensitive K^+ channels in pancreatic β-cells. Spare-channel hypothesis. *Diabetes.* 37:495–498.

6. Ashcroft, F. M., and P. Rorsman. 1989. Electrophysiology of the pancreatic β-cell. *Prog. Biophys. Mol. Biol.* 54:87–143.

7. Ashford, M. L. J. 1990. Potassium channels and modulation of insulin secretion. *In* Potassium Channels: Structure, Classification, Function and Therapeutic Potential. N. S. Cook, editor. Ellis Harwood Ltd., Chichester, England. 300–325.

8. Henquin, J. C. 1988. ATP-sensitive K^+ channels may control glucose-induced electrical activity in pancreatic B cells. *Biochem. Biophys. Res. Commun.* 156:769–795.

9. Cook, D. L., and M. Ikeuchi. 1989. Tolbutamide as mimic of glucose on β cell electrical activity: ATP-sensitive K^+ channels as common pathway for both stimuli. *Diabetes.* 38:416–421.

10. Henquin, J. C. 1990. Role of voltage- and Ca^{2+}-dependent K^+ channels in the control of glucose-induced electrical activity in pancreatic B-cells. *Pfluegers Arch. Eur. J. Physiol.* 416:568–572.

11. Trube, G., P. Rorsman, and T. Ohno-Shosaku. 1986. Opposite effects of tolbutamide and diazoxide on the ATP-dependent K^+ channel in mouse pancreatic β-cells. *Pfluegers Arch. Eur. J. Pharmacol.* 407:493–499.

12. Dunne, M. J., M. C. Illot, and O. H. Petersen. 1987. Interaction of diazoxide, tolbutamide and ATP^{4-} on nucleotide-dependent K^+ channels in an insulin-secreting cell line. *J. Membr. Biol.* 99:215–224.

13. Zünkler, B. J., S. Lenzen, K. Männer, U. Panten, and G. Trube. 1988. Concentration-dependent effects of tolbutamide, meglitinide, glipizide, glibenclamide and diazoxide on ATP-regulated K^+ currents in pancreatic B-cells. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 337:225–230.

14. Gillis, K. D., W. M. Gee, A. Hammoud, M. L. McDaniel, L. C. Falke, and S. Misler. 1989. Effects of sulfonamides on a metabolite-regulated ATP_i-sensitive K^+ channel in rat pancreatic B-cells. *Am. J. Physiol.* 257:C1119–C1127.

15. Ashcroft, S. J. H., C. J. Hedeskov, and P. J. Randle. 1970. Glucose metabolism in mouse pancreatic islets. *Biochem. J.* 118:143–154.

16. Bergsten, P., E. Gylfe, N. Wesslen, and B. Hellman. 1988. Diazoxide unmasks glucose inhibition of insulin release by counteracting entry of Ca^{2+}. *Am. J. Physiol.* 255:E422–E427.

17. Henquin, J. C., and H. P. Meissner. 1982. Opposite effects of tolbutamide and diazoxide on $^{86}Rb^+$ fluxes and membrane potential in pancreatic B-cells. *Biochem. Pharmacol.* 31:1407–1415.

18. Henquin, J. C. 1978. D-Glucose inhibits potassium efflux from pancreatic islet cells. *Nature (Lond.).* 271:271–273.

19. Meissner, H. P., and H. Schmelz. 1974. Membrane potential of beta cells in pancreatic islets. *Pfluegers Arch. Eur. J. Physiol.* 351:195–206.

20. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440–3450.

21. Grapengiesser, E., E. Gylfe, and B. Hellman. 1990. Sulfonylurea mimics the effect of glucose in inducing large amplitude oscillations of cytoplasmic Ca^{2+} in pancreatic beta-cells. *Mol. Pharmacol.* 37:461–467.

22. Hellman, B., L. A. Idahl, A. Lernmark, J. Sehlin, and I. B. Taljedal. 1974. The pancreatic β-cell recognition of insulin secretagogues. Effects of calcium and sodium on glucose metabolism and insulin release. *Biochem. J.* 138:33–46.

23. Lenzen, S., and U. Panten. 1980. 2-Oxocarboxylic acids and function of pancreatic islets in obese-hyperglycaemic mice. *Biochem. J.* 186:135–144.

24. Atwater, I., C. M. Dawson, G. T. Eddlestone, and E. Rojas. 1981. Voltage noise measurements across the pancreatic β-cell membrane: calcium channel characteristics. *J. Physiol. (Lond.).* 314:195–212.

25. Arkhammar, P., T. Nilsson, P. Rorsman, and P. O. Berggren. 1987. Inhibition of ATP-regulated K^+ channels precedes depolarization-induced increase in cytoplasmic free Ca^{2+} concentration in pancreatic β-cells. *J. Biol. Chem.* 262:5448–5454.

26. Gylfe, E. 1988. Glucose-induced early changes in cytoplasmic calcium of pancreatic β-cells studied with time-sharing dual-wavelength fluorometry. *J. Biol. Chem.* 263:5044–5048.

27. Wang, J. L., and M. L. McDaniel. 1990. Secretagogue-induced oscillations of cytoplasmic Ca^{2+} in single β and α-cells obtained from pancreatic islets by fluorescence-activated cell sorting. *Biochem. Biophys. Res. Commun.* 166:813–818.

28. Rorsman, P., and B. Hellman. 1988. Voltage-activated currents in guinea pig pancreatic α_2 cells. Evidence for Ca^{2+}-dependent action potentials. *J. Gen. Physiol.* 91:223–242.

29. Meissner, H. P., J. C. Henquin, and M. Preissler. 1978. Potassium dependence of the membrane potential of pancreatic B-cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 94:87–89.

30. Panten, U., J. Burgfeld, F. Goerke, M. Rennicke, M. Schwanstecher, A. Wallasch, B. J. Zünkler, and S. Lenzen. 1989. Control of insulin secretion by sulfonylureas, meglitinide and diazoxide in relation to their binding to the sulfonylurea receptor in pancreatic islets. *Biochem. Pharmacol.* 38:1217–1229.

31. Howell, S. L., and K. W. Taylor. 1966. Effect of diazoxide on insulin secretion in vitro. *Lancet.* i:128–129.

32. Lin, B. J., and R. E. Haist. 1973. Effects of some modifiers of insulin secretion on insulin biosynthesis. *Endocrinology.* 92:735–742.

33. Leinweber, K. P., and H. Schatz. 1982. Long-term and short-term effects of calcium, verapamil and diazoxide on biosynthesis and release of (pro-)insulin in isolated islets of rat pancreas. *Acta Endocrinol.* 99:94–100.

34. Pipeleers, D. G., M. Marichal, and W. J. Malaisse. 1973. The stimulus-secretion coupling of glucose-induced insulin release. XV. Participation of cations in the recognition of glucose by the β-cell. *Endocrinology.* 93:1012–1018.

35. Milner, R. D. G., and C. N. Hales. 1969. The interaction of various inhibitors and stimuli of insulin release studied with rabbit pancreas in vitro. *Biochem. J.* 113:473–479.

36. Henquin, J. C., S. Charles, M. Nenquin, F. Mathot, and T. Tamagawa. 1982. Diazoxide and D600 inhibition of insulin release. Distinct mechanisms explain the specificity for different stimuli. *Diabetes.* 31:776–783.

37. Malaisse, W. J., A. Sener, A. Herchuelz, and J. C. Hutton. 1979. Insulin release: the fuel hypothesis. *Metab. Clin. Exp.* 28:373–386.

38. Meglasson, M. D., and F. M. Matschinsky. 1984. New perspectives on pancreatic islet glucokinase. *Am. J. Physiol.* 246:E1–E13.

39. Velasco, J. M., J. U. H. Petersen, and O. H. Petersen. 1988. Single-channel Ba^{2+} currents in insulin-secreting cells are activated by glyceraldehyde stimulation. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 231:366–370.

40. Smith, P. A., P. Rorsman, and F. M. Ashcroft. 1989. Modulation of dihydropyridine-sensitive Ca^{2+} channels by glucose metabolism in mouse pancreatic β-cells. *Nature (Lond.).* 342:550–553.

41. Cerasi, E. 1975. Mechanisms of glucose stimulated insulin secretion in health and in diabetes: some re-evaluations and proposals. *Diabetologia.* 11:1–13.

42. Hermans, M. P., W. Schmeer, and J. C. Henquin. 1987. The permissive effect of glucose, tolbutamide and high K^+ on arginine stimulation of insulin release in isolated mouse islets. *Diabetologia.* 30:659–665.