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Research Article

Glucose stimulation of insulin release involves closure of ATP-sensitive K+ channels (K(+)-ATP channels), depolarization, and Ca2+ influx in B cells. However, by using diazoxide to open K(+)-ATP channels, and 30 mM K to depolarize the membrane, we could demonstrate that another mechanism exists, by which glucose can control insulin release independently from changes in K(+)-ATP channel activity and in membrane potential (Gembal et al. 1992. J. Clin. Invest. 89:1288-1295). A similar approach was followed here to investigate, with mouse islets, the nature of this newly identified mechanism. The membrane potential-independent increase in insulin release produced by glucose required metabolism of the sugar and was mimicked by other metabolized secretagogues. It also required elevated levels of cytoplasmic Cai2+, but was not due to further changes in Cai2+. It could not be ascribed to acceleration of phosphoinositide metabolism, or to activation of protein kinases A or C. Thus, glucose did not increase inositol phosphate levels and hardly affected cAMP levels. Moreover, increasing inositol phosphates by vasopressin or cAMP by forskolin, and activating protein kinase C by phorbol esters did not mimic the action of glucose on release, and down-regulation of protein kinase C did not prevent these effects. On the other hand, it correlated with an increase in the ATP/ADP ratio in islet cells. We suggest that the membrane potential-independent control of [...]



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Mechanisms by Which Glucose Can Control Insulin Release Independently from Its Action on Adenosine Triphosphate–sensitive K⁺ Channels in Mouse B Cells

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Abstract

Glucose stimulation of insulin release involves closure of ATPsensitive K⁺ channels (K⁺-ATP channels), depolarization, and Ca²⁺ influx in B cells. However, by using diazoxide to open K⁺-ATP channels, and 30 mM K to depolarize the membrane, we could demonstrate that another mechanism exists, by which glucose can control insulin release independently from changes in K⁺-ATP channel activity and in membrane potential (Gembal et al. 1992. J. Clin. Invest. 89:1288-1295). A similar approach was followed here to investigate, with mouse islets, the nature of this newly identified mechanism. The membrane potential-independent increase in insulin release produced by glucose required metabolism of the sugar and was mimicked by other metabolized secretagogues. It also required elevated levels of cytoplasmic Ca_i²⁺, but was not due to further changes in Ca_i²⁺. It could not be ascribed to acceleration of phosphoinositide metabolism, or to activation of protein kinases A or C. Thus, glucose did not increase inositol phosphate levels and hardly affected cAMP levels. Moreover, increasing inositol phosphates by vasopressin or cAMP by forskolin, and activating protein kinase C by phorbol esters did not mimic the action of glucose on release, and down-regulation of protein kinase C did not prevent these effects. On the other hand, it correlated with an increase in the ATP/ADP ratio in islet cells. We suggest that the membrane potential-independent control of insulin release exerted by glucose involves changes in the energy state of B cells. (J. Clin. Invest. 1993. 91:871-880.) Key words: adenine nucleotides • calcium • insulin secretion • pancreatic islets • stimulus-secretion coupling

Introduction

The mechanisms by which glucose controls pancreatic B cell function are complex (1-4). A number of studies have established that a metabolic control of ionic events in B cells is a critical event in stimulus-secretion coupling (4-9), and as-

signed to ATP-sensitive K^+ channels $(K^+$ -ATP channels)¹ in the plasma membrane a pivotal role in this control (10-14). It is now unanimously accepted that the entry of glucose in B cells is followed by an acceleration of metabolism that generates one or several signals which close these K⁺-ATP channels. The resulting decrease in K⁺ conductance leads to depolarization of the membrane with subsequent opening of voltage-dependent Ca²⁺ channels. Ca²⁺ influx through these channels then increases, leading to a rise in cytoplasmic Ca²⁺, which eventually activates an effector system responsible for exocytosis of insulin granules. The essential role of K⁺-ATP channels is strikingly illustrated by the inhibition of insulin release brought about by diazoxide. This drug selectively opens K⁺-ATP channels (15, 16) and, thereby, causes repolarization of the plasma membrane (17) and arrest of Ca^{2+} influx (17–19), although the metabolism of glucose is unabated (19, 20).

Recently, however, we presented evidence that glucose can control insulin release independently from its action on K⁺-ATP channels (21). The existence of this mechanism could be established by opening K⁺-ATP channels with diazoxide, but restoring Ca²⁺ influx by depolarizing the membrane with high extracellular K⁺. Under these conditions glucose still caused a concentration-dependent increase of insulin release (21). In the present study we have investigated the possible mechanisms underlying this membrane potential-independent way of regulating insulin release.

Methods

Solutions. The medium used was a bicarbonate-buffered solution which contained 120 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, and 24 mM NaHCO₃. It was gassed with O_2/CO_2 (94:6) to maintain pH 7.4 and was supplemented with bovine serum albumin (1 mg/ml). Ca²⁺-free solutions were prepared by replacing CaCl₂ with MgCl₂ and addition of 100 μ M EGTA. When the concentration of KCl was increased to 30 mM, that of NaCl was decreased accordingly to maintain isoosmolarity.

Measurements of insulin release. 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 a first type of experiments, the islets were first preincubated for 60 min in a control medium containing 15 mM glucose. The islets were then placed in batches of 25 in parallel perifusion chambers and perifused at a flow rate of 1.25 ml/min (22). Effluent fractions were collected at 2-min intervals, and insulin was measured by a double-antibody radioimmunoassay with rat insulin as the standard (Novo Research Institute, Bagsvaerd, Denmark).

In another type of experiments, after the initial preincubation of 60

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^{1.} Abbreviations used in this paper: AVP, arginine vasopressin; K⁺-ATP channels, ATP-sensitive K⁺ channels; KIC, α -ketoisocaproate; α PDD, 4 α -phorbol 12,13-didecanoate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

min the islets were distributed in 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.

Measurements of cAMP. After the initial preincubation of 60 min in a control medium, batches of 12 islets were incubated for 60 min in 1 ml of medium containing appropriate concentrations of glucose and test substances. At the end of the incubation the tubes were briefly centrifuged and 0.95 ml of medium withdrawn and saved for insulin assay. The medium was immediately replaced by 0.95 ml of ice-cold acetate buffer containing 0.25 mM isobutyl-methylxanthine. Islet cAMP content was then determined with a commercially available kit (Du Pont-New England Nuclear, Boston, MA) as described previously (23).

Measurements of ATP and ADP. After the initial preincubation of 60 min in a control medium, batches of five islets were incubated for 60 min in 0.4 ml of medium containing the appropriate concentrations of glucose and test substances. The incubations were stopped by addition of 0.6 ml of ice-cold trichloracetic acid to a final concentration of 5%. The tubes were then vortexed, left at room temperature for 15 min, and centrifuged for 5 min in a microfuge (Beckman Instruments, Inc., Palo Alto, CA). A fraction (400 μ l) of the supernatant was then thoroughly mixed with 1.5 ml of diethyl ether, and the ether phase containing trichloracetic acid was discarded. This step was repeated three times to ensure complete elimination of trichloracetic acid. The extracts were then diluted with 400 µl of a buffer containing 40 mM Hepes, 3 mM MgCl₂, and KOH to adjust pH at 7.75. They were then frozen at -20°C until the day of the assay. ATP was assayed by a luminometric method (24). An aliquot (50 μ l) of each sample was mixed with 190 μ l of the above buffer and 60 μ l of a commercially available ATP monitoring reagent containing firefly luciferase and luciferin (LKB-Wallac, Turku, Finland). The emitted light was measured in a luminometer (Biocounter M 2000, Lumac, Landgraaf, The Netherlands). Another 50- μ l aliquot of each sample was first incubated for 30 min with 190 μ l of buffer containing 1.5 mM phosphoenol pyruvate and 2.3 U/ml pyruvate kinase to convert ADP into ATP. ATP was then assayed as described above and ADP content was calculated by difference. Appropriate blanks, ATP, and ADP standards were run through the entire procedure including the extraction step. All measurements were made in duplicates.

Measurements of inositol phosphates. After isolation, the islets were loaded with $[2-^{3}H]myo$ -inositol (The Radiochemical Centre, Amersham, UK) during a preincubation of 2 h in the presence of 15 mM glucose. After washing, batches of 20–25 islets were then incubated for 60 min in 1 ml of medium containing 1 mM inositol, 5 mM LiCl, and appropriate concentrations of glucose and test substances. At the end of the incubation, 0.1 ml of the medium was taken for insulin measurement before addition to each tube of 3 ml of CHCl₃/CH₃OH/concentrated HCl (200:100:1, vol/vol/vol) and 100 μ l of EDTA (100 mM). Free inositol and inositol phosphates contained in the islets were then separated by anion exchange chromatography (25), as described previously (26). In this study, however, all inositol phosphates were eluted together, by adding 16 ml of a mixture of 1 M ammonium formate and 0.1 M formic acid to the columns.

Measurements of cytosolic Ca^{2+} . After isolation, the islets were cultured for 1-2 d in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 100 IU/ml of penicillin and 100 μ g/ml of streptomycin. The concentration of glucose was 10 mM. Cytosolic Ca^{2+} was measured by microspectrofluorimetry in whole islets loaded with fura-2 (Molecular Probes, Inc., Eugene, OR). The tissue was excited successively at 340 and 380 nm and the fluorescence emitted at 510 nm was captured by a CCD camera (Photonic Science Ltd., Tunbridge Wells, UK). The images were analyzed with the system Magical (Applied Imaging, Sunderland, UK). The technique was as previously described (21) except that the time interval between ratioed images was 9 instead of 12 s. Down-regulation of protein kinase C (PKC). After isolation, the islets were cultured for 20–22 h in RPMI medium containing 5.5 mM glucose and supplemented with either 200 nM phorbol 12-myristate 13-acetate (PMA) or 200 nM of the inactive phorbol ester 4α -phorbol 12,13-didecanoate (α -PDD). After washing and preincubation for 30 min in a control medium, the islets were incubated, in batches of 3, in 1 ml of medium containing appropriate concentrations of glucose and test substances. At the end of the incubation, an aliquot of the medium was taken for insulin assay. Insulin content of the islets was also determined, for each batch, after extraction in 0.5 ml of acid ethanol.

Materials. Diazoxide was obtained from Schering-Plough Avondale (Rathdrum, Ireland); deoxyglucose, D-glyceraldehyde, mannoheptulose, cycloheximide, PMA, and α -PDD were obtained from Sigma Chemical Co. (St. Louis, MO); dibutyryl (db)-cAMP and α -ketoisocaproate (KIC) were obtained from Aldrich Chemie (Steinhein, FRG); arginine vasopressin (AVP) was obtained from Peninsula Laboratories Inc. (Belmont, CA); forskolin was obtained from Calbiochem-Behring Corp. (San Diego, CA); ATP, ADP, phosphoenolpyruvate, and pyruvate kinase were from Boehringer (Mannheim, FRG), and all other reagents were obtained from Merck A.G. (Darmstadt, FRG).

Presentation of results. All results are presented as means±SEM for the indicated number of experiments or of batches of islets (obtained in the indicated number of experiments). The statistical significance of differences between means was assessed by analysis of variance followed by a test of Dunnett when several experimental groups are compared with a control group, or by a test of Newman-Keuls when multiple comparisons are made. In a few instances, when only two groups were involved, a test of t was used. Differences were considered significant at P < 0.05.

Results

Characteristics of the effects of glucose on K-induced insulin release. Perifused islets were used to characterize the effects of various glucose concentrations on insulin release induced by 30 mM K in the presence of 250 μ M diazoxide. As shown in Fig. 1, control release in normal K was low in all glucose concentrations, which attests that the normal effect of glucose is fully blocked by diazoxide. Increasing the concentration of K to 30 mM rapidly triggered insulin release. Both the pattern and the amplitude of this response to high K were influenced by the prevailing glucose concentration. In the absence of glucose, the rate of insulin release increased rapidly and markedly (by about 13-fold) before progressively returning to basal values. In the presence of 3 or 6 mM glucose, K-induced insulin release was biphasic. The initial response was smaller than that seen in a glucose-free medium, but was followed by a secondary increase, which was more sustained in 6 mM than in 3 mM glucose (Fig. 1). When the concentration of glucose was 10 mM or higher (up to 30 mM), the pattern of insulin release was no longer clearly biphasic, but the rate of increase was still higher during the initial minutes than later.

When the concentration dependency of the effects of glucose was computed by integrating total insulin release over the 60-min period of perifusion with high K, the dose-response curve displayed two components: a first increase between 0 and 6 mM, significant at 3 mM (P < 0.05 by analysis of variance and Dunnett's test), and a second increase > 6 mM (Fig. 2 A). This curve contrasts with the control, sigmoidal relationship between insulin release and the concentration of glucose in a perifusion medium containing 4.8 mM K and no diazoxide. Half-maximal stimulation of insulin release was produced by 10 mM glucose in high K and diazoxide, and by 15.5 mM glucose under control conditions.



When total insulin release was integrated over the first 10 min, a clear inhibitory effect of 3 and 6 mM glucose (P < 0.01) was evidenced, whereas the response was not significantly affected by 1 mM glucose or by concentrations of 10 mM and higher (Fig. 2 *B*). Similar results were obtained by computing the rate of release at 4 and 6 min (not shown). The curve



Figure 2. Concentration dependency of the effects of glucose on insulin release by perifused mouse islets stimulated with 30 mM K in the presence of 250 μ M diazoxide (•). The experiments were similar to those illustrated in Fig. 1. Insulin release was integrated over the indicated periods of stimulation with 30 mM K. Control experiments (\circ) were performed with a medium containing 4.8 mM K, no diazoxide and the same glucose concentration throughout. Insulin release was integrated over the same time periods as for the experiments with high K. Values are the mean±SEM (when larger than the symbol) for six or seven experiments.

Figure 1. Effects of glucose and of KIC on the stimulation of insulin release by 30 mM K in mouse islets perifused with a medium containing diazoxide. The experiments lasted 110 min, but only the last 10 min of the initial equilibration period are shown. The concentration of glucose (G 0–G 20 mM) and of KIC (2 mM) remained constant throughout. Diazoxide (250 μ M) was also present from the start of the experiment. The concentration of K was increased from 4.8 to 30 mM for 60 min as indicated. Values are the mean±SEM for six or seven experiments.

characterizing the effect of glucose during the last 50 min of stimulation still showed two components (Fig. 2 C). It, however, tended to an hyperbolic shape for the last 10 min of stimulation (Fig. 2 D). During this last period, half-maximal stimulation was achieved by 9.5 mM glucose, in contrast to 15 mM glucose under control conditions (low K and no diazoxide).

Role of cytoplasmic Ca_i^{2+} . The concentration of cytoplasmic Ca_i^{2+} in B cells was estimated in whole islets loaded with the Ca indicator, fura-2. At the start of the experiments, when the concentration of K was 4.8 mM, Ca_i^{2+} was low and similar in 0, 6, and 20 mM glucose (55±4, 65±5, and 63±5 nM, respectively; n = 9), because of the presence of diazoxide. When the concentration of K was raised to 30 mM, a biphasic increase in Ca_i^{2+} occurred, with an initial peak that was higher (P < 0.01) in 0 glucose than in 6 mM glucose (Fig. 3). During steady-state stimulation with 30 mM K, Ca_i^{2+} remained stable in 6 mM glucose, but slowly and regularly increased with time in the absence of glucose. The changes in Ca_i^{2+} measured in the pres-



Figure 3. Effect of glucose (G) on the increase in cytoplasmic Ca²⁺ brought about in islet cells by 30 mM K. The concentration of glucose was constant and diazoxide (250 μ M) was present throughout the experiments, whereas the concentration of K was increased from 4.8 to 30 mM as indicated by the arrow. The traces correspond to the mean response (±SEM) obtained in nine islets.

ence of 20 mM glucose were similar to those in 6 mM glucose (not shown). At the peak, Ca_i^{2+} averaged (n = 9) 314±17, 236±8, and 244±7 nM in 0, 6, and 20 mM glucose, respectively. After 30 min of stimulation with high K, it averaged 346±13, 199±7, and 212±7 nM in 0, 6, and 20 mM glucose, respectively. When the experiments were performed in a Cafree medium, high K did not increase Ca_i^{2+} in the absence or presence of glucose (not shown).

We also verified that the effect of glucose on insulin release was not altered in cultured islets loaded with fura-2. Loading with the Ca indicator had no effect on the insulin response to high K in the presence of diazoxide and absence of glucose $(4.0\pm0.1 \text{ vs. } 4.2\pm0.2 \text{ ng/islet per 60 min, } n = 3)$. This also did not prevent 6 mM glucose from reducing the initial response by 30% and increasing the late one by 150%.

Effects of various agents. Fig. 1 shows that 2 mM KIC affected insulin release induced by 30 mM K in the presence of

Table I. Effects of Various Agents on Insulin Release by Mouse Islets Incubated in the Presence of 30 mM K and 250 μ M Diazoxide

Experimental conditions			
Glucose	Test substance	Insulin release	
тM	mM	percentage of controls	
Series A			
0	—	100±3 (60)	
20	—	281±11* (60)	
0	Deoxyglucose 20	101±8 (16)	
0	Galactose 20	91±6 (16)	
0	Glyceraldehyde 10	203±12* (20)	
0	Pyruvate 20	101±5 (14)	
0	Lactate 20	102±7 (12)	
0	Glutamine 20	149±12* (12)	
0	Leucine 20	253±20* (12)	
0	Glutamine 10 + leucine 10	293±22* (16)	
0	Inositol 10	97±6 (12)	
0	Azide 2	51±4* (16)	
0	NH₄Cl 1	65±7* (14)	
0	NH₄Cl 5	32±3* (14)	
0	No CaCl ₂	14±2* (12)	
Series B			
20	_	100±3 (32)	
20	Mannoheptulose 20	50±3* (21)	
20	Cycloheximide 0.05	99±5 (12)	
20	Azide 2	54±3* (21)	
20	NH₄Cl I	77±4* (12)	
20	NH₄Cl 5	50±3* (17)	
20	No CaCl ₂	4±1* (12)	

Batches of 3 islets were incubated for 1 h in 1 ml of medium containing 30 mM K and 250 μ M diazoxide. The medium also contained the indicated concentration of glucose and of test substance. Results are expressed as a percentage of the average release of insulin by control islets within the same experiments. In series A, control release in 0 mM glucose amounted to 7.2±0.3 ng/islet per h. In series B, control release in 20 mM glucose amounted to 19.8±1.0 ng/islet per h. Values are means±SEM for the indicated number of batches of islets, obtained in at least three separate experiments. * P < 0.01 vs. controls without test substance at the same glucose concentration (Analysis of variance followed by test of Dunnett). diazoxide in a similar way as 6 mM glucose. Compared to the response measured in a glucose-free medium without KIC, the initial release (0-10 min) was decreased by 60% (P < 0.001 by t test), whereas the late response (10-60 min) was increased more than threefold.

The effects of other agents were tested with islets incubated for 1 h in the presence of 30 mM K and 250 μ M diazoxide (Table I). Deoxyglucose, galactose, pyruvate, lactate, and inositol were ineffective, whereas glyceraldehyde, and glutamine and leucine, alone or in combination, increased insulin release.

The stimulatory effect of glucose was unaffected by cycloheximide, which suggests that synthesis of new proteins is not required. On the other hand, it was decreased by mannoheptulose and azide, which respectively inhibit glucose phosphorylation by glucokinase and interfere with mitochondrial respiration. Azide also inhibited insulin release in the absence of glucose (Table I).

The effects of NH_4Cl were tested because glucose raises cytoplasmic pH in B cells (27), and because alkalinization of B cells with NH_4Cl increases insulin release under certain conditions (28). NH_4Cl was rather found to cause a dose-dependent inhibition of K-induced insulin release in the absence and presence of 20 mM glucose. Finally, omission of extracellular Ca abolished insulin release in 0 and 20 mM glucose, as already reported (21).

Role of cAMP. The cAMP concentration of islets incubated for 1 h in a medium containing 0 glucose, 30 mM K, and 250 μ M diazoxide amounted to 12.0±1.0 fmol/islet (n = 18). It was not significantly affected by 3 or 6 mM glucose, and marginally increased (P < 0.05) by 10 and 20 mM glucose (Fig. 4).



Figure 4. Effects of glucose and of forskolin $(1 \mu M)$ on cAMP levels in and insulin release by mouse islets stimulated by 30 mM K in the presence of 250 μ M diazoxide. Batches of 12 islets were incubated for one hour in a medium containing the indicated concentration of glucose with or without forskolin. At the end of the incubation, insulin was measured in the medium and cAMP in the tissue. Values are the mean±SEM for 18 batches of islets from six separate experiments.

This weak effect of glucose contrasted with the strong increase brought about by 1 μ M forskolin: twofold in the absence of glucose and fourfold in 6 mM glucose. There was no correlation between islet cAMP concentrations and insulin release. Insulin release was similarly increased by 6 mM glucose and by forskolin in the absence of glucose, but cAMP levels were about 65% higher in the latter situation. Moreover, insulin release was 25% higher (P < 0.01) in 20 mM glucose than in 6 mM glucose plus forskolin although cAMP levels were 4.4-fold lower.

Given the opposite effects of certain glucose concentrations on the early and late phases of insulin release induced by 30 mM K in the presence of diazoxide, the effects of forskolin were also tested in perifused islets. Fig. 5 shows that forskolin did not alter the time course of release, but simply shifted the rate of release to higher values. In particular, it did not decrease the initial response and increase the late one as does glucose. The membrane permeant db-cAMP had no significant effect in the absence of glucose, but mimicked those of forskolin in 6 mM glucose (Fig. 5).



Figure 5. Effects of forskolin $(1 \mu M)$ and db-cAMP (0.5 mM) on the stimulation of insulin release by 30 mM K in mouse islets perifused with a medium containing diazoxide. The experiments lasted 110 min, but only the last 10 min of the initial equilibration period are shown. The concentration of glucose (G 0-G 6 mM) remained constant throughout. Diazoxide (250 μ M) and forskolin or db-cAMP were also present from the start of the experiment. Values are the mean±SEM for five experiments, except with db-cAMP where the values are the mean for two experiments.



Figure 6. Effects of glucose and of AVP (100 nM) on inositol phosphate levels in and insulin release by mouse islets stimulated by 30 mM K in the presence of 250 μ M diazoxide. Batches of 20-25 islets were incubated for one hour in a medium containing the indicated concentration of glucose with or without AVP, and 5 mM LiCl. At the end of the incubation, insulin was measured in the medium and inositol phosphate levels in the tissue. Values are the mean±SEM for 12 batches of islets from six separate experiments, except for the groups with AVP where values are the mean for eight batches of islets.

Role of phosphoinositides and PKC. To test whether the effects of glucose on insulin release involve changes in phosphoinositide turnover, the islets were labeled with $[2-^{3}H]$ myoinositol before being incubated in the presence of high K, diazoxide, 5 mM lithium, and various concentrations of glucose. Their content in total inositol phosphates was then determined. As shown in Fig. 6, inositol phosphate levels were not affected by any concentration of glucose under the present experimental conditions, although the sugar produced its usual dose-dependent stimulation of insulin release. This sharply contrasts with the large increase in inositol phosphate levels brought about by 100 nM AVP, which, however, did not modify insulin release. Interestingly, the effect of AVP on inositol phosphates was larger (P < 0.01) in 6 mM glucose than in the glucose-free medium.

Stimulation of PKC by 25 nM PMA increased total K-induced insulin release by 50% (P < 0.05) in the absence of glucose, by 65% (P < 0.01) in 3 mM glucose, and by about 300% in 6 mM glucose. This potentiation did not substantially alter the time course of release (Fig. 7).

To evaluate further a possible role of PKC in the stimulation by glucose, the effects of the sugar were tested in islets which had been cultured for 20–22 h in the presence of a high concentration of PMA, a process widely used to down-regulate PKC. Islets cultured in parallel with the inactive phorbol ester α -PDD served as controls. Because the presence of PMA in the culture medium markedly stimulates insulin release the islet insulin content was determined in each batch of islets. Table II thus presents results as absolute amounts of insulin release and



Figure 7. Effects of PMA (25 nM) on the stimulation of insulin release by 30 mM K in mouse islets perifused with a medium containing diazoxide. The experiments lasted 110 min, but only the last 10 min of the initial equilibration period are shown. The concentration of glucose (G 0–G 6 mM) remained constant throughout. Diazoxide (250 μ M) was also present from the start of the experiment. Values are the mean±SEM for five experiments. The controls were the same as those in Fig. 4.

as a percentage of the insulin content, at the start of the incubation.

Experimental series A served to establish that down-regulation of PKC was achieved. After culture of the islets with α -PDD, 15 mM glucose stimulated insulin release 11-fold, compared to 0 glucose. This effect was strongly potentiated by 25 nM PMA or by 100 nM mezerein, an activator of PKC structurally different from phorbol esters. In islets cultured with PMA, glucose stimulated insulin release almost to the same extent (ninefold) but this represented a much larger fractional release of the hormone. On the other hand, the stimulatory effect of glucose was no longer potentiated by PMA or mezerein (Table II).

When islets cultured for 20 h with α -PDD were incubated in the presence of 30 mM K and 250 μ M diazoxide, glucose produced its normal, concentration-dependent increase in insulin release, and 25 nM PMA strongly amplified the effect of glucose. Down-regulation of PKC by culture with PMA did not prevent glucose from increasing insulin release under these conditions, but abolished the acute amplification of this effect by 25 nM PMA. The absolute changes in insulin release induced by glucose were smaller than in islets treated with α -PDD but the fractional changes were larger (Table II).

Role of adenine nucleotides. Islet ATP and ADP levels were measured after 60 min of incubation in a medium containing 30 mM K, 250 μ M diazoxide, and various concentrations of glucose. ATP levels increased when the concentration of glucose increased from 0 to 10–20 mM, whereas ADP levels did not significantly change (Fig. 8). This resulted in a concentration-dependent increase in the ATP/ADP ratio, that was doubled by 20–30 mM glucose. Half-maximal increase occurred at 11 mM glucose. An excellent correlation was found between the ATP/ADP ratio measured in islets at the end of the 60 min of incubation, and the amount of insulin released during the last 10 min of the one-hour perifusion with high K, diazoxide and various concentrations of glucose (Fig. 9 A).

The influence of other agents on ATP and ADP levels was

Table II. Effects of Down-Regulation of PKC on Insulin Release by Incubated Mouse Islets

	Islets cultured with a-PDD			Islets cultured with PMA		
Incubation conditions	Insulin content Insulin		ulin release	Insulin content	Insulin release	
	ng/islet	ng/islet per h	percentage of content	ng/islet	ng/islet per h	percentage of content
Series A: 4.8 mM K						
Glucose 0	235±12	0.39±0.05	0.17±0.02	60±5	0.76±0.11	1.31±0.21
Glucose 15 mM	248±8	4.40±0.46 [‡]	1.79±0.20 [‡]	59±3	6.93±0.53 [‡]	11.9±1.10 [‡]
G 15 mM + PMA 25 nM	227±8	27.9±2.4 [‡]	12.5±1.26 [‡]	70±7	7.29±0.46 [‡]	11.0±0.92 [‡]
G 15 mM + Mezerein 100 nM	227±8	16.2±0.8 [‡]	7.17±0.32 [‡]	58±4	6.74±0.72 [‡]	12.0±1.35 [‡]
Series B: 30 mM K + diazoxide						
Glucose 0	243±7	15.7±0.9	6.53±0.35	73±4	5.11±0.30	7.17±0.43
Glucose 10 mM	249±14	19.0±0.9*	7.81±0.36*	73±4	7.06±0.53*	10.0±0.76*
G 10 mM + PMA 25 nM	236±10	35.0±1.5 [‡]	14.9±0.42 [‡]	67±3	6.68±0.55*	9.94±0.64*
Glucose 20 mM	243±7	24.4±1.1 [‡]	10.1±0.44 [‡]	66±3	8.54±0.62 [‡]	13.2±1.00 [‡]

The islets were first cultured for 20–22 h in RPMI medium containing 5.5 mM glucose and supplemented with either 200 nM α -PDD or 200 nM PMA. The islets were then incubated for 1 h under the indicated conditions. At the end of the incubation, an aliquot of the medium was taken for measurement of insulin release and the islet insulin content was determined after extraction in acid ethanol. The presented insulin content corresponds to the sum of the insulin content at the end of the incubation plus the amount of insulin released during that incubation. Values are means±SEM for 8 batches of islets from two cultures in series A and for 20 batches of islets from five cultures in series B. * P < 0.05, * P < 0.01 vs. controls without glucose in the same series (analysis of variance followed by test of Dunnett).

also studied (Table III). In the absence of glucose, deoxyglucose slightly decreased ATP levels; galactose, pyruvate, and lactate were without effect; azide decreased the ATP/ADP ratio; glyceraldehyde and the combination glutamine plus leucine increased ATP levels and the ATP/ADP ratio. On the other hand, the effects of 20 mM glucose were inhibited by mannoheptulose, azide, and NH₄Cl. A good correlation was found between the effects of these various agents on the ATP/ADP ratio in the islets and on insulin release (Fig. 9 *B*). A notable exception was the omission of extracellular Ca which abolished insulin release in 0 and 20 mM glucose (Table II) without affecting the ATP/ADP ratio in islet cells (Table III).

Discussion

The present study extends our recent demonstration (21) 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. It also suggests that this property of glucose may be linked to changes in the energy state of B cells, while excluding several other possible mechanisms.

In our previous experiments performed with incubated islets we observed that the concentration dependency of the effect of glucose on insulin release in the presence of high K and diazoxide displayed a peculiar biphasic shape, with an increase already at 2 mM glucose. A similar curve was again obtained here, when perifused islets were stimulated for 60 min with high K in different glucose concentrations. These dynamic experiments also revealed an unsuspected complexity of the effect of glucose on the time-course of release. Whereas the steady-state response progressively increased with the glucose concentration, the rapid response was paradoxically decreased by low glucose concentrations. The dose–response curve is thus the sum of an inhibitory component detected at 3 and 6 mM glucose, and a stimulatory component observed at all glucose concentrations. It is possible, however, that the inhibitory com-



Figure 8. Effects of various concentrations of glucose on ATP and ADP levels and on the ATP/ADP ratio in mouse islets stimulated by 30 mM K in the presence of 250 μ M diazoxide. Batches of five islets were incubated for 1 h in the presence of the indicated concentration of glucose. Values are the mean±SEM for 25 batches of islets from five separate experiments.



Figure 9. Correlations between insulin release and the ATP/ADP ratio in mouse islets stimulated by 30 mM K in the presence of 250 μ M diazoxide. (A) Experiments performed with various concentrations of glucose. Values are taken from Fig. 2 D for insulin release and Fig. 8 for the ATP/ADP ratio. (B) Experiments performed in the presence of various agents. Values are taken from Table I for insulin release and Table III for the ATP/ADP ratio. The conditions are: a, G 0; b, G 0 and 20 mM deoxyglucose; c, G 0 and 20 mM galactose; d, G 0 and 10 mM glyceraldehyde; e, G 0 and 20 mM glutamine and leucine; f, G 0 and 2 mM azide; g, G 0 and 20 mM lactate; h, G 0 and 20 mM pyruvate; i, G 20 mM; j, G 20 mM and 20 mM mannoheptulose; k, G 20 mM and 2 mM azide; l, G 20 mM and 5 mM NH₄Cl. Values are shown as the mean±SEM.

ponent does not disappear at 10 mM glucose and above, but is masked by the fast development of the stimulatory component. An acute inhibitory effect of glucose on insulin release has been observed previously with islets perifused with a medium containing diazoxide and a normal K concentration (19). It has been ascribed to the ability of the sugar to promote Ca^{2+} sequestration in the absence of Ca^{2+} influx. Our observation that 6 mM glucose attenuated the rapid increase in cytoplasmic Ca_i^{2+} brought about by high K is consonant with this hypothesis which, admittedly, requires more direct testing.

Elimination of the inhibitory component made the doseresponse curve more though not completely hyperbolic, thus accentuating the differences with the sigmoidal relationship between the glucose concentration and insulin release under control conditions. A significant effect of glucose was observed at 3 mM and the half-maximal response at 9–10 mM, as compared with more than 6 and 15 mM in control islets.

When islets are incubated for 60 min, only the stimulatory component of the glucose action can be reliably studied. It was inhibited by mannoheptulose, which inhibits glucose phos-

able III. Effects of Various Agents on A	TP and ADP Levels in Mouse Islets Incubate	ed in the Presence of 30 mM K and 250 µM Diazoxide
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Experimental conditions				
Glucose	Test substance	АТР	ADP	ATP/ADP
mM	mM	pmol/islet	pmol/islet	
Series A				
0	_	7.1±0.2	4.4±0.1	1.61±0.03
0	Deoxyglucose 20	6.3±0.2*	4.2±0.2	1.52±0.05
0	Galactose 20	7.3±0.3	4.3±0.1	1.70±0.04
0	Pyruvate 20	7.5±0.2	4.4±0.2	1.76±0.10
0	Lactate 20	7.2±0.3	4.5±0.2	1.64±0.09
0	Glyceraldehyde 10	9.5±0.5 [‡]	4.2±0.1	2.23±0.08 [‡]
0	Glutamine 10 + leucine 10	13.6±0.5 [‡]	4.0±0.2	3.47±0.19 [‡]
0	Azide 2	6.3±0.3*	4.6±0.2	1.39±0.04*
0	No CaCl ₂	8.3±0.4*	5.1±0.3*	1.65±0.03
Series B				
20	_	12.6±0.3	4.0±0.1	3.24±0.10
20	Mannoheptulose 20	7.7±0.3 [‡]	4.0±0.2	1.95±0.08 [‡]
20	Azide 2	7.5±0.4 [‡]	5.0±0.2 [‡]	1.47±0.03 [‡]
20	NH₄Cl 5	9.6±0.6 [‡]	4.5±0.3	2.14±0.09 [‡]
20	No CaCl ₂	13.0±0.4	4.4±0.1	2.96±0.13

Batches of 5 islets were incubated for 1 h in 0.4 ml of medium containing 30 mM K and 250 μ M diazoxide. The medium also contained the indicated concentration of glucose and test substance. Values are means±SEM for 55 (series A) or 40 (series B) batches of control islets without test substance and of 15 batches of islets with each test substance. *P < 0.05, ‡P < 0.01 vs controls without substance but at the same glucose concentration (Analysis of variance followed by test of Dunnett).

phorylation (29, 30) and by azide, which poisons mitochondria (31); it was reproduced by glyceraldehyde, leucine with glutamine, and KIC, which are all well metabolized (32); it was not mimicked by 2-deoxyglucose, galactose, pyruvate, or lactate, which are not or only poorly metabolized in islet cells (29, 30). It is thus clear that the stimulatory effect of glucose is linked to an increase in B cell metabolism.

Omission of extracellular Ca prevented the rise in Ca_i^{2+} and the stimulation of insulin release produced by high K both in the absence or presence of glucose. The stimulatory effect of glucose on insulin release is thus Ca^{2+} dependent. It, however, does not result from a further rise in Ca_i^{2+} . Steady-state Ca_i^{2+} (in the presence of high K) was similar in 20 and 6 mM glucose, whereas the rate of insulin release was twice higher in the former situation. Moreover, Ca_i^{2+} was higher in 0 glucose than in 6 or 20 mM glucose, but insulin release was much smaller. There was also no correlation between the time course of the changes in Ca_i^{2+} and insulin release. In the absence of glucose, Ca_i^{2+} slowly increased with time, whereas the rate of insulin release decreased. It is thus obvious that glucose, somehow, modulates the effectiveness of Ca_i^{2+} on the secretory machinery.

The modest increase in islet cAMP concentration that glucose causes is not the triggering signal of insulin release under physiological conditions (4, 33, 34), but cAMP is known to sensitize the releasing system to Ca^{2+} . Participation of PK A in the phenomenon studied here was thus plausible. However, no correlation was found between islet cAMP levels and insulin release in the presence of various concentrations of glucose, with or without forskolin. We, therefore, conclude that the membrane potential-independent effect of glucose on insulin release is not secondary to an increase in cAMP levels, but we cannot exclude the possibility that activation of PKA by basal cAMP levels participates in the effects of glucose. Nutrient insulin secretagogues slightly stimulate inositol phosphate formation in insulin-secreting cells (35, 36). This effect is, at least partly, due to activation of phospholipase C by the influx of Ca^{2+} that the secretagogues cause. Under our experimental conditions, glucose did not affect inositol phosphate levels while increasing insulin release. Conversely, strong stimulation of phosphoinositide breakdown by AVP (26) did not augment insulin release. We, therefore, suggest that the membrane potential-independent effect of glucose is not due to an acceleration of phosphoinositide turnover.

The role of PKC in glucose-induced insulin release is still controversial, but the balance negates (37-40) rather than supports (41) its importance. However, because PKC is believed to sensitize the releasing machinery to Ca²⁺, its involvement in the effects of glucose studied here was possible. This does not seem to be the case, for PMA was only poorly active on K-induced insulin release unless glucose was present, and never gave to insulin release the pattern produced by an increase in glucose concentration. This clearly shows that an effect of glucose other than an activation of PKC plays a preeminent role, but does not totally excludes the participation of PKC. To test further this possibility, islets were pretreated overnight with PMA, a technique widely used to down-regulate PKC (37-40). The inability of PMA and of mezerein to increase insulin release acutely after such treatment attests that down-regulation of PKC was achieved. This, however, did not prevent glucose from stimulating insulin release under control conditions, or in the presence of high K and diazoxide. We, therefore, conclude that neither the effect of glucose under physiological conditions, nor its membrane potential-independent effect, more specifically studied here, is critically dependent on PKC.

Glucose increases ATP levels (42-45) and the ATP/ADP ratio (44, 45) in isolated islets studied under physiological conditions. These observations support the hypothesis that, by

modulating the activity of K+-ATP channels, the changes in cytoplasmic ATP/ADP ratio constitute a major link between glucose metabolism and the control of B cell membrane potential. They, however, neither prove this hypothesis nor exclude the intervention of other coupling factors. Thus, a stimulation of insulin release by the perfused rat pancreas, without detectable changes in the ATP/ADP ratio in islet cells, was recently observed upon acute stimulation with glucose in the presence of amino acids (46). In the present experiments (made under nonphysiological conditions) glucose was found to cause a dose-dependent increase in the ATP/ADP ratio in islet cells, and this increase correlated very well with the changes in insulin release brought about by the sugar. Moreover, an excellent correlation was also found between insulin release in the presence of various agents (active ones, alone and with inhibitors, or inactive ones) and the ATP/ADP ratio in the tissue. The only exception was the absence of extracellular Ca, which totally blocked release without affecting the ATP/ADP ratio.

Under our experimental conditions, K⁺-ATP channels are no longer operative as they are maintained widely open by the high concentration of diazoxide. We, therefore, suggest that there exists another step of stimulus-secretion coupling where adenine nucleotides play an important role. The fact that activation of PKA by cAMP or of PKC by PMA was poorly effective in the absence of glucose in face of the high concentration of cytosolic Ca_i^{2+} indicates that this step is rather distal in the chain of events leading to exocytosis of insulin granules. Two possibilities are that ATP is simply required to sustain an energy-dependent phenomenon (e.g., the movement of granules) or that a minimum concentration of ATP is necessary for adequate phosphorylation of critical targets. Both are compatible with the observation that forskolin and PMA become effective in the presence of glucose, but do not readily explain why the correlation between insulin release and the ATP/ADP ratio extends over the whole range of glucose concentrations. An alternative is that another, yet unidentified, substance, the concentration of which varies in parallel with the ATP/ADP ratio, is the true coupling factor.

In conclusion, the mechanism by which glucose can control insulin release independently from changes in K⁺-ATP channel activity and changes in B cell membrane potential requires stimulation of B cell metabolism, but does not involve changes in cytoplasmic Ca_i^{2+} , cAMP levels, inositol phosphate levels, or PKC activity. On the other hand changes in the energy state of B cells may be involved. The possibility is raised that the ATP/ ADP ratio (or a related messenger) controls insulin release by regulating both the activity of K⁺-ATP channels and a much later step of stimulus-secretion coupling. At the latter level, it appears to amplify the effectiveness of the rise in Ca_i^{2+} that follows the primary action of glucose on K⁺-ATP channels and membrane potential. An important challenge will be to establish the relative contribution of defects at both levels of control in the abnormalities of glucose-induced insulin release in B cells of non-insulin-dependent diabetic patients.

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