The K^+-ATP Channel–Independent Pathway of Regulation of Insulin Secretion by Glucose

In Search of the Underlying Mechanism

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By closing ATP-sensitive K^+ (K^+-ATP) channels, glucose promotes depolarization-dependent Ca^{2+} entry and cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) rise in β-cells. Ca^{2+}-dependent exocytosis of insulin granules is then potentiated by a K^+-ATP channel–independent action of glucose. The underlying mechanisms of this second pathway are still unclear. They were studied by incubating normal mouse islets in the presence of diazoxide to open K^+-ATP channels and 30 mmol/l K^+ to restore Ca^{2+} entry. The effect of glucose did not require priming of β-cells by preincubation in the presence of high glucose and could not be attributed to interaction of the sugar with a "glucoreceptor." There is no evidence that protein kinases A and C are involved in the K^+-ATP channel–independent pathway, because inhibitors of the kinases did not alter the effect of glucose. In 3 mmol/l glucose, fatty acids did not influence K^+-induced insulin secretion, even in the presence of bromopalmitate, an inhibitor of fatty acid oxidation. Bromopalmitate alone had no effect, but it decreased the potentiation that the fatty acids produce in 20 mmol/l glucose. It is thus unlikely that long-chain acyl CoAs mediate the effect of glucose. The action of glucose was not associated with an increase in arachidonic acid release from the islets and was not mimicked by exogenous arachidonic acid. Phospholipase A_2 inhibitors antagonized the effect of glucose, but their action was not reversed by arachidonic acid or palmitate and was associated with a fall in islet ATP. No evidence could be found for the intervention of NO, cGMP, Mg, phosphate, phosphatidylinositol 3-kinase, or pertussis toxin–sensitive G-proteins. Formycin A, an adenosine analog that is converted to formycin A–triphosphate in islets, increased insulin secretion in the absence and presence of glucose. In conclusion, the present and our previous results strongly suggest that among all known potential second messengers, adenine nucleotides are the best candidates as regulators of insulin secretion through the K^+-ATP channel–independent pathway. ***Diabetes*** **47:1713–1721, 1998**

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$AACOCF_3$, arachidonyl trifluoromethyl ketone; ACA, *N*-(*p*-amylcinnamoyl)anthranilic acid; AVP, arginine vasopressin; BIM, bisindolylmaleimide; Br-cGMP, 8-bromoguanosine 3′,5′-cyclic monophosphate; BSA, bovine serum albumin; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; Cal-C, calphostin-C; DHS, dihydrosphingosine; FCS, fetal calf serum; HELSS, haloenol lactone suicide substrate; IP, inositol phosphate; K^+-ATP, ATP-sensitive K^+ channel; L-NAME, Nω-nitro-L-arginine methylester; L-NMMA, N^G-methyl-L-arginine; PKA, protein kinase A; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; PNP-α-Glu, *p*-nitrophenyl-α-D-glucopyranoside; PNP-β-Glu, *p*-nitrophenyl-β-D-glucopyranoside; PNP-galactose, *p*-nitrophenyl-α-D-galactopyranoside; PNP-Glu, *p*-nitrophenyl-D-glucopyranoside; PTX, pertussis toxin.

The secretion of insulin, like that of other hormones, is controlled by neurotransmitters and hormones that, upon binding to β-cell membrane receptors, activate classical transduction pathways culminating in the stimulation of protein kinase A (PKA) and protein kinase C (PKC) (1–4). However, β-cells differ from other secretory cells in at least two respects: they are fuel sensors that adapt their biological response to variations in the plasma concentration of glucose and other nutrients, and they do so through changes in the metabolism of these nutrients (1,2,5,6).

The primary and best-characterized pathway of the stimulation of insulin secretion by glucose serves to produce the triggering signal of exocytosis, a rise in the cytoplasmic concentration of Ca^{2+} ($[Ca^{2+}]_i$) through the following sequence of events (7–10). Glucose metabolism leads to closure of ATP-sensitive K^+ channels (K^+-ATP channels), thereby causing membrane depolarization, opening of voltage-dependent Ca^{2+} channels, and acceleration of Ca^{2+} influx.

A second pathway of regulation by glucose has been identified more recently, by treating β-cells with diazoxide to hold K^+-ATP channels open and with high extracellular K^+ to depolarize the membrane and restore Ca^{2+} influx (11,12). Under these conditions, glucose does not increase $[Ca^{2+}]_i$ but amplifies the action of the ion on the releasing process (11,13). The existence of this K^+-ATP channel–independent pathway is now widely accepted (14–18), but the underlying mechanisms are still unclear. These mechanisms were investigated here in experiments designed to test the possible role of a number of messengers that have been suggested to play a role in the regulation of insulin secretion.

RESEARCH DESIGN AND METHODS

Solutions and preparation. The control medium used for islet isolation and preincubation was a bicarbonate-buffered solution that contained 120 mmol/l NaCl, 4.8 mmol/l KCl, 2.5 mmol/l $CaCl_2$, 1.2 mmol/l $MgCl_2$, and 24 mmol/l $NaHCO_3$. It was gassed with O_2/CO_2 (94:6) to maintain pH 7.4 and was supplemented with 1 mg/ml bovine serum albumin (BSA). The test medium used to study the K^+-ATP channel–independent effect of glucose on insulin secretion contained 30 mmol/l KCl and only 94.8 mmol/l NaCl. It was supplemented with 250 µmol/l diazoxide, a concentration that does not affect glucose metabolism in mouse islets (19). Some experimental protocols required modifications of the solutions that are described in the legend to the appropriate figures.

Islets were isolated by collagenase digestion of the pancreas of fed female NMRI ice (25–30 g), followed by hand-picking. They were used immediately after isotion except in one experiment in which they were first cultured for 42 h in RPMI 640 medium (Flow Laboratories, ICN Biomedicals, Irvine, U.K.) containing 10 mmol/l glucose, 10% heat-inactivated fetal calf serum (FCS), 100 IU/ml penicillin, nd 100 µg/ml streptomycin, without or with 200 ng/ml pertussis toxin (PTX). In nother experiment, the islets were cultured for 20 h to load them with H]arachidonic acid (see below).

tudy of insulin secretion. The islets were preincubated for 60 min in a control medium containing 3 mmol/l glucose (15 mmol/l in a few experiments, which e specified in RESULTS) before being distributed into batches of three. When ATP nd ADP were also measured, the islets were distributed into batches of five. Each atch of islets was then incubated for 60 min in 1 ml of medium containing various concentrations of glucose and test substances. At the end of the incubation, portion of the medium was withdrawn and appropriately diluted for insulin easurement by a double-antibody radioimmunoassay with rat insulin as the standard (Novo Research Institute, Bagsvaerd, Denmark).

leasurement of islet ATP and ADP. Islet ATP and ADP determinations were ade at the end of incubations that also served to study insulin secretion. After e aliquot (0.625 ml) for insulin measurement was taken, the islets were incubated r another 5 min. Tubes were kept at 37°C during the whole procedure. The incuation was stopped by the addition of 0.125 ml of trichloroacetic acid to a final oncentration of 5%. Tubes were then treated, and the ATP and ADP contents of e islets were measured, as published previously (20).

leasurement of [^{3}H]arachidonic acid release. The method used to measure H]arachidonic acid release was similar to that described by Konrad et al. (21). riefly, islets were labeled with [^{3}H]arachidonic acid during 20 h of culture in RPMI edium (as above except that the concentration of glucose was 5 mmol/l and that FCS only 2.5%) supplemented with 5 µCi [^{3}H]arachidonic acid per milliliter. After ashing, the islets were preincubated for 60 min in a control medium containing mmol/l glucose before being distributed into batches of 10 and transferred into cubation tubes containing 1 ml of medium with appropriate test substances. After) min, 100 µl were taken for insulin assay, 800 µl for measurement of released H]arachidonic acid, and the islets were recovered for determination of their [^{3}H] ontent. [^{3}H]arachidonic acid release was then expressed as a percentage of the acer content of the same islets.

laterials. Diazoxide was a gift of Schering-Plough Avondale (Rathdrum, Ireland). TP, ADP, and all reagents for their measurements were from Boehringer-annheim (Mannheim, Germany). *p*-Nitrophenyl-α-D-glucopyranoside (PNP-α-lu), *p*-nitrophenyl-β-D-glucopyranoside (PNP-β-Glu), *p*-nitrophenyl-α-D-galacpyranoside (PNP-galactose), phorbol 12-myristate 13-acetate (PMA), arachidonic id, acetylcholine chloride, N^G-methyl-L-arginine acetate salt (L-NMMA), Nω-nitro-arginine methylester (L-NAME), 8-bromoguanosine 3′,5′-cyclic monophosnate (Br-cGMP), and PTX were from Sigma (St. Louis, MO). Rp-cAMPS was from BI (Natick, MA). Forskolin, bisindolylmaleimide (BIM), calphostin-C (Cal-C), *N*-(2-amylcinnamoyl)anthranilic acid (ACA), and arachidonyl trifluoromethyl etone ($AACOCF_3$) were from Calbiochem (San Diego, CA). Dihydrosphingosine DHS), wortmannin, and LY294002 were from Biomol (Hamburg, Germany). almitate, stearate, oleate, and 2-bromopalmitate were from Aldrich Chemie teinheim, Germany). Formycin A was from Fluka Chemie (Bornem, Belgium). H]arachidonic acid was from Amersham (Bucks, U.K.).

resentation of results. Results are presented as means ± SE for the indicated umber of batches of islets from the indicated number of separate experiments. he statistical significance for differences between means was assessed by analys of variance, followed by a test of Dunnett when several experimental groups e compared with a control group, or by a test of Newman-Keuls for multiple comarisons. Differences were considered significant at $P < 0.05$.

ESULTS AND DISCUSSION

ole of priming. The first reports of the existence of a K⁺-TP channel–independent effect of glucose on insulin secreon were based on experiments using mouse islets preincuated in the presence of 15 mmol/l glucose (11) and rat islets reincubated in the presence of 3 mmol/l glucose (12). The uestion was raised whether priming might be necessary to bserve this effect in the mouse (22), although the phenomnon of priming is not prominent in that species (23). Figure shows results obtained in islets preincubated in a control nedium containing a low or a high concentration of glucose nd then incubated in a medium with high K⁺ and diazoxide. Glucose produced a concentration-dependent increase in nsulin secretion in both groups. The response was 50–100%

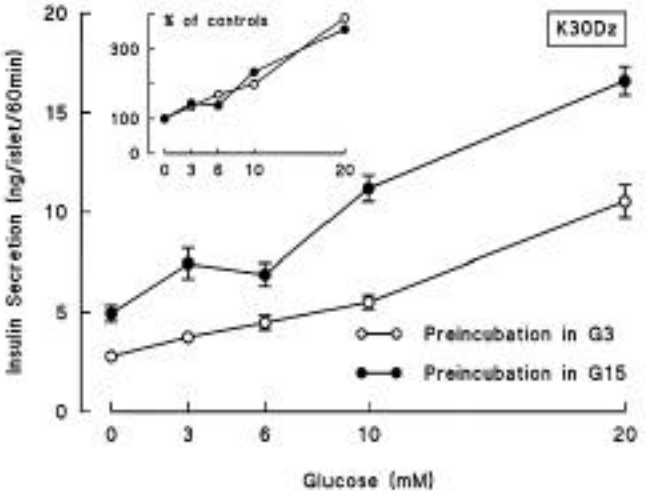


FIG. 1. Influence of the preincubation conditions on the K⁺-ATP channel–independent effect of glucose on insulin secretion. Islets were first preincubated for 60 min in a control medium containing 3 (○) or 15 (●) mmol/l glucose (G). They were then distributed in batches of three in 1 ml of medium containing 30 mmol/l K⁺, 250 µmol/l diazoxide, and the indicated concentration of glucose. At the end of the incubation, an aliquot of the medium was taken for insulin assay. Values are means ± SE for 25 batches of islets from five separate experiments. The inset shows the same results expressed as a percentage of controls in the absence of glucose.

larger ($P < 0.05$) after preincubation in high than in low glucose, but the relative increase produced by each concentration of glucose was similar (Fig. 1, inset). These results show that some priming also exists in mouse β-cells but is not required for the K⁺-ATP channel–independent effect of glucose to be expressed.

Role of a putative glucoreceptor. *p*-Nitrophenyl-D-glucopyranoside (PNP-Glu) is an inhibitor of the sweet taste receptor (24). The α-anomer has been reported to inhibit glucose-induced insulin secretion without affecting glucose metabolism, whereas PNP-β-Glu was inactive (25). The observation that 10 mmol/l PNP-α-Glu inhibited the K⁺-ATP channel–independent effect of glucose in rat islets prompted the suggestion that this pathway might be initiated by the interaction of glucose itself with a glucoreceptor (14). Our results do not confirm this proposal as they show that PNP-α-Glu increased insulin secretion in the presence of high K⁺, independently of the presence or absence of glucose (Table 1). A similar effect was produced by PNP-β-Glu and by PNP-galactose, which even casts doubts on the mode of action of these compounds. Our conservative conclusion is that PNP-α-Glu is not a suitable tool to establish the intervention of a putative glucoreceptor in the K⁺-ATP channel–independent action of glucose. In contrast, strong evidence has been presented that the metabolism of the sugar by β-cells is essential to produce that effect (11,13,15).

Role of PKA. We previously concluded that the cAMP-PKA pathway was unlikely to be involved in the K⁺-ATP channel–independent effect of glucose on the basis of two types of observations (13). First, exogenous dibutyryl cAMP and activation of adenylylcyclase with forskolin had little effect on insulin secretion induced by high K⁺ in the absence of glucose. Second, in the presence of 6 mmol/l glucose and high K⁺, the fivefold increase in cAMP induced by forskolin stimulated insulin secretion to a lesser extent than did a rise of the glucose

TABLE 1
Influence of glucoreceptor ligands on the K^+-ATP channel–independent effect of glucose on insulin secretion

Test agent (10 mmol/l)	Insulin secretion (ng · islet^{-1} · 60 min^{-1})		
	Glucose (0 mmol/l)	Glucose (3 mmol/l)	Glucose (15 mmol/l)
—	3.8 ± 0.3	4.0 ± 0.3	6.8 ± 0.4
PNP-α-glucose	5.9 ± 0.5	5.8 ± 0.4	9.3 ± 0.8
PNP-β-glucose	5.9 ± 0.6	6.2 ± 0.5	9.0 ± 1.0
PNP-galactose	5.5 ± 0.5	6.0 ± 0.6	8.9 ± 0.7

Data are means ± SE for 15 or 20 batches of islets. After preincubation for 60 min in a control medium containing 3 mmol/l glucose, batches of three islets were incubated in a medium containing 30 mmol/l K^+, 250 µmol/l diazoxide, and the indicated concentration of glucose. The medium was also supplemented with 10 mmol/l PNP-α-glucose, PNP-β-glucose, or PNP-galactose.

concentration to 20 mmol/l, although the latter barely raised islet cAMP. To test further the possible participation of the cAMP pathway, we used here Rp-cAMPS, a PKA antagonist (26). Rp-cAMPS decreased K^+-induced insulin secretion in low and high glucose, even in the absence of forskolin (Fig. 2), which suggests that basal PKA activity contributes to the control secretory response. In islets treated with Rp-cAMPS, no significant effect of forskolin on insulin secretion was observed. In contrast, the increase brought about by 20 mmol/l glucose persisted, and its relative amplitude was similar to that occurring in control islets (3.7 vs. 3.6-fold) (Fig. 2). In conclusion, the present and our previous data (13) conclusively establish that the K^+-ATP channel–independent effect of glucose is not mediated by the cAMP-PKA pathway.

Role of PKC. In our previous study using mouse islets (13), four arguments led to the conclusion that the phospholipase C (PLC)-PKC pathway was not implicated in the K^+-ATP channel–independent effect of glucose. First, direct activation of PKC with PMA was poorly effective on insulin secretion induced by high K^+ in the absence of glucose. Second, glucose did not increase islet inositol phosphate (IP) levels in the presence of high K^+. Third, arginine vasopressin (AVP) increased IP levels without potentiating K^+-induced insulin secretion. Fourth, the effect of glucose on insulin secretion persisted after downregulation of PKC (13).

Our conclusion was challenged by others because the PKC inhibitor staurosporin decreased the effect of glucose on K^+-induced insulin secretion from rat islets (14). Because staurosporin has a poor specificity (27), we tested here the effects of three other PKC inhibitors. As compared with 3 mmol/l glucose, 20 mmol/l glucose increased high K^+-induced insulin secretion about 2.5-fold; a similar effect was produced by the addition of 25 nmol/l PMA to 3 mmol/l glucose (Fig. 3). BIM and DHS, two PKC inhibitors (28,29) were ineffective in the presence of low or high glucose alone but partially counteracted the effect of PMA. Cal-C, another PKC inhibitor (30), decreased control insulin secretion by ~25% and attenuated the action of PMA to a larger extent than that of 20 mmol/l glucose (55 vs. 25%) (Fig. 3).

Another argument against our conclusion was that our failure to detect an effect of glucose on IPs (13) could be due to our labeling of the islets with [^{3}H]inositol in the presence of high glucose (15). This objection is correct. When we labeled the islets in the presence of 3 instead of 15 mmol/l glucose and then measured IP production in the presence of high K^+, a small increase by glucose was observed (J.C. Jonas, J.-C.H., unpublished observations). However, the effect was small, not exceeding 50%, as compared with ~300% for the effect of AVP (13). A direct comparison of the two species (31) showed that IP production from islets incubated in the presence of diazoxide, high K^+, and high glucose is insignificantly increased in the mouse (~25%) as compared with the rat (~300%). Thus, there seems to be agreement that the K^+-ATP channel–independent effect of glucose is not mediated by the PLC-PKC pathway in mouse β-cells. Whether this pathway is involved in the rat is possible (31), but this conclusion should not rest on the use of staurosporin only.

Role of long-chain acyl CoAs. Cytosolic long-chain acyl CoAs are thought to serve as key second messengers in glucose-induced insulin secretion, but their site of action has yet to be identified (32). Because they open K^+-ATP channels and therefore tend to cause repolarization (33), their positive action on secretion cannot be at the early step of membrane depolarization. On the other hand, an effect distal to the rise in $[Ca^{2+}]_i$ would make them potential mediators of the K^+-ATP channel–independent action of glucose. Because exogenous

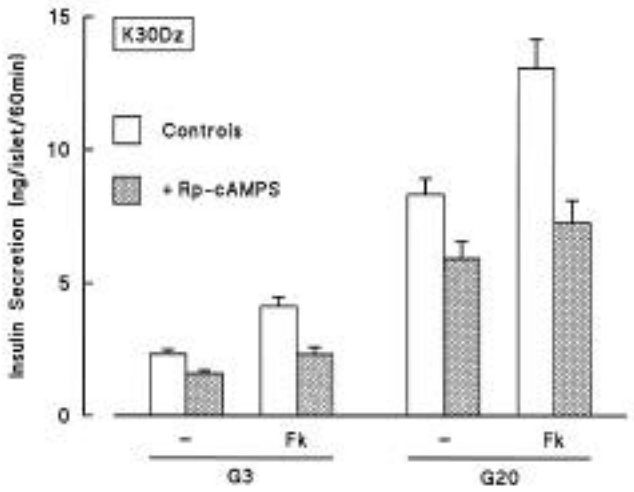
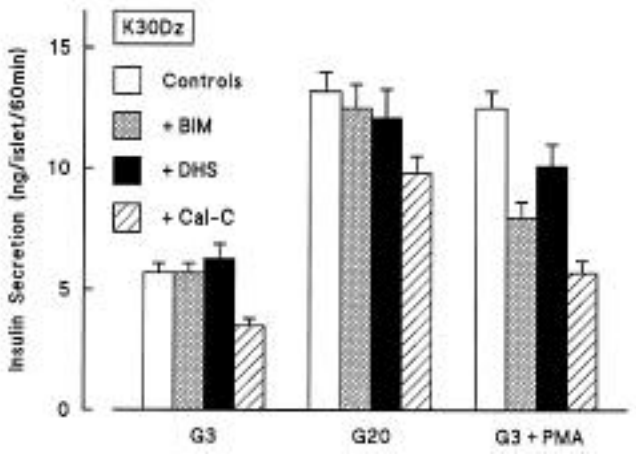


FIG. 2. Influence of a PKA inhibitor on the K^+-ATP channel–independent effect of glucose on insulin secretion. Islets were first preincubated for 30 min in a control medium containing 15 mmol/l glucose. They were then distributed in batches of three in 0.4 ml of control medium containing 3 mmol/l glucose alone or with 500 µmol/l of Rp-cAMPS. After 60 min, 0.1 ml of concentrated test medium was added to reach final concentrations of 30 mmol/l K^+ and 100 µmol/l diazoxide in all tubes, and either 3 mmol/l glucose (G3) or 20 mmol/l glucose (G20) with or without 0.1 µmol/l forskolin (Fk) as indicated. After 60 min of incubation, an aliquot of the medium was taken for insulin assay. Values are means ± SE for 15 batches of islets from three separate experiments.



IG. 3. Influence of PKC inhibitors on the K⁺-ATP channel–indepen-ent effect of glucose on insulin secretion. Islets were first preincu-ated for 60 min in a control medium containing 3 mmol/l glucose. They ere then distributed in batches of three in 1 ml of medium contain-ıg 30 mmol/l K⁺, 250 µmol/l diazoxide, and either 3 mmol/l glucose G3), 20 mmol/l glucose (G20), or 3 mmol/l glucose + 25 nmol/l PMA G3+PMA). The medium also contained, as indicated, 1 µmol/l BIM, 5 mol/l DHS, or 1 µmol/l Cal-C. After 60 min of incubation, an aliquot f the medium was taken for insulin assay. Values are means ± SE for 5–25 batches of islets from three to five separate experiments.

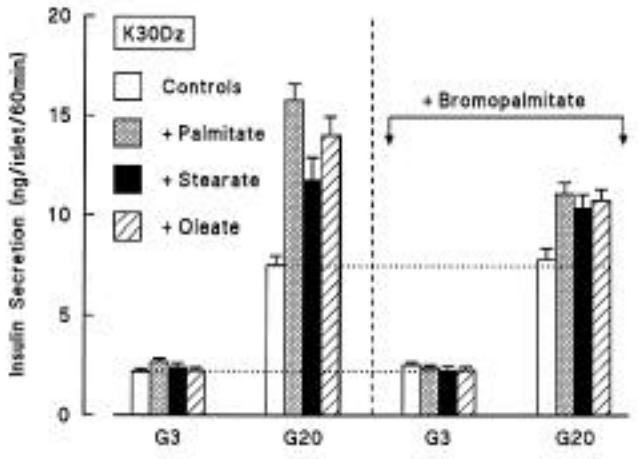


FIG. 4. Influence of long-chain fatty acids on the K⁺-ATP channel–independent effect of glucose on insulin secretion. Islets were first preincubated for 60 min in a control medium containing 3 mmol/l glucose. They were then distributed in batches of three in 1 ml of medium containing 30 mmol/l K⁺, 250 µmol/l diazoxide, and either 3 mmol/l glucose (G3) or 20 mmol/l glucose (G20), with or without 500 µmol/l of the indicated fatty acids. The concentration of BSA was 80 µmol/l (molar ratio fatty acid:BSA = 6.25). When 500 µmol/l bromopalmitate was also added, the concentration of BSA was doubled to maintain the same fatty acid:BSA ratio. After 60 min of incubation, an aliquot of the medium was taken for insulin assay. Values are means ± SE for 20 batches of islets from four separate experiments.

atty acids increase long-chain acyl CoAs in islets (33), we ested the ability of palmitate, stearate, and oleate to mimic ne effect of glucose on insulin secretion in the presence of igh K^+ (Fig. 4). They were ineffective in low glucose but aug-ıented insulin secretion when combined with 20 mmol/l lucose. To ascertain that this glucose dependency did not eflect the requirement of a high glucose concentration for ıhibition of oxidation and stimulation of esterification of atty acids (34), we also tested the effects of bromopalmitate. his analog is not metabolizable and inhibits fatty acid oxi-ation by inactivating carnitine-palmitoyl transferase I (35).

Bromopalmitate alone did not influence insulin secretion t 3 or 20 mmol/l glucose. It also failed to disclose any stim-latory effect of palmitate, stearate, and oleate in low glucose nd inhibited the potentiation by palmitate and oleate in 20 mol/l glucose (Fig. 4). The effect of the fatty acids thus eems to depend on their metabolism in β-cells. It should also e borne in mind that, unlike a rise in the glucose concen-ation, palmitate increased $[Ca^{2+}]_i$ in islet cells perifused vith 30 mmol/l K^+ and diazoxide (36). In conclusion, unless xogenous fatty acids exert unknown effects (32) that would ounteract those of long-chain acyl CoAs issued from ndogenous fatty acids, it is unlikely that long-chain acyl oAs mediate the K^+-ATP channel–independent effect of glu-ose on insulin secretion.

ole of a phospholipase A_2 (PLA_2)-arachidonic acid athway. Several lines of evidence point to arachidonic acid s a potential second messenger in glucose-induced insulin ecretion, which could act by both facilitating Ca^{2+} influx and ncreasing the action of Ca^{2+} on exocytosis (37–39). Arachi-onic acid can be released from membrane phospholipids by a^{2+}-dependent PLA_2 (40,41) or by an ATP-stimulated Ca^{2+}-ndependent PLA_2 (42). The latter enzyme in particular is a lausible mediator of the K^+-ATP channel–independent action f glucose, which is associated with an increase in the TP:ADP ratio and no further rise of $[Ca^{2+}]_i$ in β-cells (13).

As a first approach, we investigated whether glucose increases arachidonic acid release from islets prelabeled with [^{3}H]arachidonic acid, and we used a muscarinic agonist as a positive control (43). In the presence of both normal and high K^+, 1 µmol/l acetylcholine stimulated [^{3}H]arachidonic acid release (Fig. 5). High glucose was ineffective alone but potentiated the action of acetylcholine at least in the presence of normal K^+ ($P < 0.001$). Overall, there was no correlation between the changes in [^{3}H]arachidonic acid and insulin release (Fig. 5). Thus, in both types of medium, acetylcholine increased [^{3}H]arachidonic acid release but stimulated insulin release in the presence of high glucose only. Conversely, glucose stimulated insulin release without increasing [^{3}H]arachidonic acid release. However, these results, based on a radiochemical approach, do not exclude the possibility that a PLA_2 was stimulated by glucose and that the levels of unesterified arachidonic acid increased within islet cells, as was shown by direct mass measurements in rat islets (44).

The effects of exogenous arachidonic acid were thus tested. In a control medium containing 4.8 mmol/l KCl, no diazoxide, and 14.5 µmol/l BSA, 100 µmol/l arachidonic acid marginally increased basal insulin secretion in 3 mmol/l glucose (0.19 ± 0.03 vs. 0.08 ± 0.01 ng · islet^{-1} · h^{-1}) and potentiated the response to 15 mmol/l glucose (6.67 ± 0.67 vs. 3.67 ± 0.63 ng · islet^{-1} · h^{-1}; $n = 15$, $P < 0.01$). Under these conditions, where the arachidonate:BSA ratio was 6.9, the increase in insulin release did not reflect any damage of the islets because it was abrogated by epinephrine (data not shown). In a test medium containing 30 mmol/l K^+ and 250 µmol/l diazoxide, arachidonic acid decreased insulin secretion in the presence of 3 mmol/l glucose by about 35% ($P < 0.01$) but did not affect that measured in the presence of 20 mmol/l glucose (Fig. 6). In rat islets, 20 µmol/l arachidonic acid without BSA increased insulin release induced by 20 mmol/l K^+ but not that by higher concentrations (44).

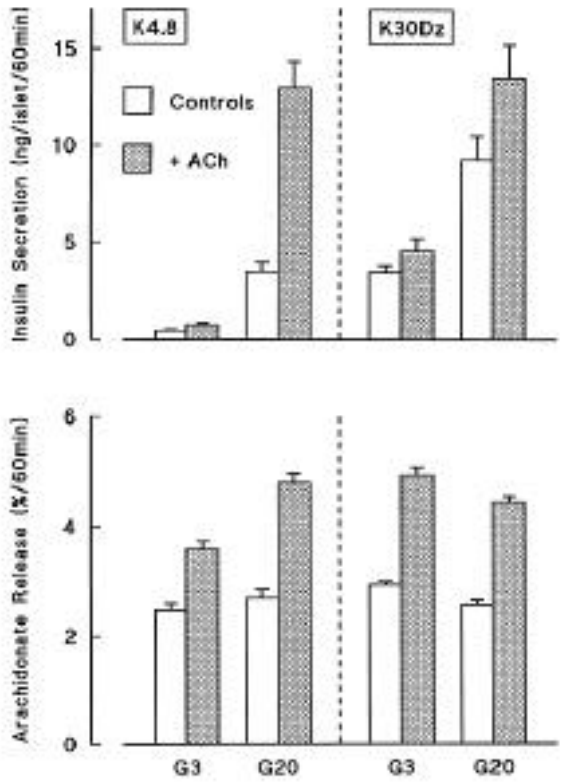


FIG. 5. Comparison of the effect of glucose on insulin secretion and arachidonic acid release. After loading with [^{3}H]arachidonic acid during 20 h of culture, the islets were washed and preincubated for 60 min in a control medium containing 3 mmol/l glucose. They were then distributed in batches of 10 in a control medium containing 4.8 mmol/l K^+ or in a test medium containing 30 mmol/l K^+ and 250 µmol/l diazoxide. The concentration of glucose (G) was 3 or 20 mmol/l, and acetylcholine (1 µmol/l) was present or not, as indicated. After 60 min of incubation, aliquots of the medium were taken for insulin assay. [^{3}H]arachidonic acid was measured in another aliquot, and the amount of tracer released over 60 min was expressed as a percentage of the islet content. The [^{3}H]arachidonic-acid incorporation averaged 6,895 ± 171 cpm per islet. Values are means ± SE for 12–18 batches of islets from four to six separate experiments.

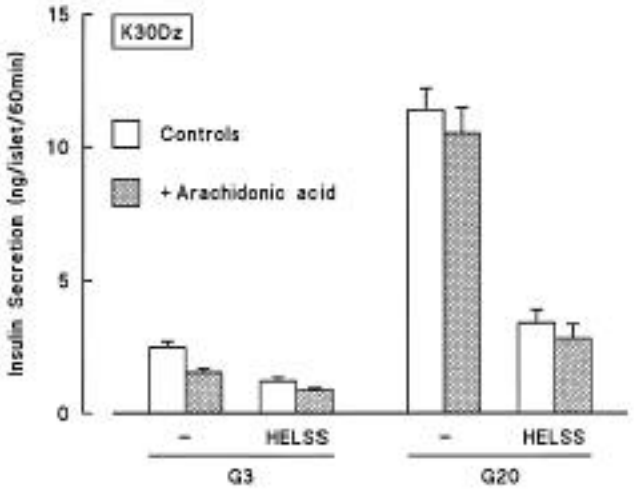


FIG. 6. Influence of arachidonic acid on the K^+-ATP channel–independent effect of glucose on insulin secretion. Islets were preincubated for 60 min in a control albumin-free medium containing 3 mmol/l glucose alone or with 5 µmol/l HELSS. They were then distributed in batches of three in 1 ml of medium containing 30 mmol/l K^+, 250 µmol/l diazoxide, 5 mmol/l HEPES, and 3 mmol/l glucose (G3), 20 mmol/l glucose (G20), with or without 100 µmol/l arachidonic acid. The medium also contained 14.5 µmol/l BSA (molar ratio arachidonic acid:BSA = 6.9). HELSS was thus present only during the preincubation of the indicated group. Arachidonic acid was dissolved in 100 mmol/l Na_2CO_3/25% ethanol and diluted 500 times in the medium. This solvent was also added to the control medium. After 60 min of incubation, an aliquot of the medium was taken for insulin assay. Values are means ± SE for 10–25 batches of islets from two to five separate experiments.

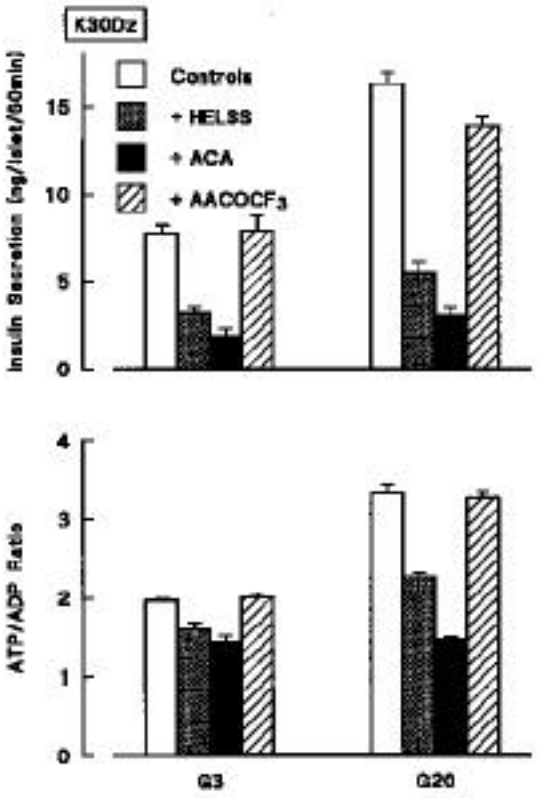
Next, the effects of PLA_2 inhibitors were evaluated. In rat islets treated with 3–25 µmol/l haloenol lactone suicide substrate (HELSS), an inhibitor of the ATP-activated PLA_2 (42), glucose-induced $[Ca^{2+}]_i$ rise and insulin secretion were inhibited, but KCl-induced $[Ca^{2+}]_i$ rise was not impaired (45). Figure 6 shows that preincubation of the islets with 5 µmol/l HELSS inhibited insulin secretion induced by 30 mmol/l K^+ by 50 and 70% in 3 and 20 mmol/l glucose, respectively. The inhibitory effect of HELSS was not reversed by exogenous arachidonic acid (Fig. 6), which should have occurred if the sole action of HELSS was to decrease arachidonic acid production by PLA_2.

ACA and $AACOCF_3$ are two other inhibitors of PLA_2 that have been found to inhibit glucose-induced insulin secretion (21,46). Their effects were compared with those of HELSS on secretion and the ATP:ADP ratio in islets incubated with high K^+ (Fig. 7). $AACOCF_3$ was ineffective on insulin secretion and adenine nucleotides. HELSS and ACA inhibited K^+-induced secretion in 3 and 20 mmol/l glucose, but they also decreased the ATP:ADP ratio in the same islets. The latter effect was unexpected because of reports that HELSS and ACA did not impair glucose oxidation by isolated rat islets (21,45).

Because $AACOCF_3$ inhibits an arachidonate-selective PLA_2 (41,47,48), whereas HELSS and ACA also inhibit PLA_2 that hydrolyzes palmitate-containing substrates (42,49,50), we eventually evaluated whether exogenous palmitate could reverse the inhibitory effects of the latter two drugs. The results are shown in Fig. 8. As reported above, palmitate potentiated K^+-induced insulin secretion in the presence of high glucose only. The inhibitory effect of ACA was unaffected by palmitate, but that of HELSS was partly reversed by the fatty acid at least in the presence of 20 mmol/l glucose. However, the relative decrease in insulin secretion caused by HELSS was the same (69%) without or with palmitate.

In conclusion, there is no evidence that stimulation of a PLA_2 underlies the K^+-ATP channel–independent effect of glucose. Thus, glucose did not stimulate arachidonic acid release under these conditions, while the stimulation by acetylcholine was accompanied by an increase in insulin release only in the presence of high glucose. Moreover, exogenous arachidonate did not mimic the effects of glucose. Finally, inhibitors of PLA_2 were found to exert, in this system at least, untoward effects on the ATP:ADP ratio, which may be sufficient to explain their inhibitory effect on secretion.

Role of NO and cGMP. Islets contain a constitutive Ca^{2+}/calmodulin-dependent NO synthase, but the role of NO in glucose-induced insulin secretion is controversial (51,52). Neither NMMA nor NAME, two inhibitors of NO synthase (53), mimicked or impaired the effect of glucose on insulin secretion in the presence of high K^+ (Fig. 9), which, therefore, cannot be ascribed to inhibition or stimulation of NO synthase. Glucose and NO increase cGMP levels in islets, but the significance of this increase for insulin secretion is unclear (51,54). Neither in low nor high glucose did Br-cGMP influence insulin secretion



IG. 7. Influence of PLA_2 inhibitors on the K^+-ATP channel–indepen-ent effect of glucose on insulin secretion and the ATP:ADP ratio. slets were preincubated for 60 min in a control albumin-free medium ontaining 3 mmol/l glucose alone or with 5 µmol/l of HELSS. They ere then distributed in batches of five in 1 ml of medium containing mg/ml BSA, 30 mmol/l K^+, 250 µmol/l diazoxide, and 3 mmol/l glucose G3) or 20 mmol/l glucose (G20). As indicated, the medium was also upplemented with 100 µmol/l ACA or 100 µmol/l $AACOCF_3$. HELSS as thus present only during the preincubation of the indicated roup. After 60 min of incubation, the medium was taken for insulin ssay, and islets were processed for nucleotides measurement. Values re means ± SE for 10 batches of islets from two separate experiments.

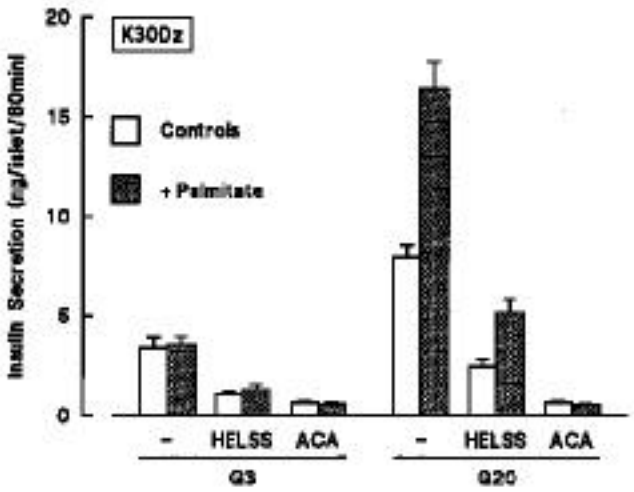


FIG. 8. Influence of palmitate on the inhibition of the K^+-ATP channel–independent effect of glucose by HELSS and ACA. Islets were preincubated for 60 min in a control albumin-free medium containing 3 mmol/l glucose alone or with 5 µmol/l HELSS. They were then distributed in batches of three in 1 ml of medium containing 30 mmol/l K^+, 250 µmol/l diazoxide, and either 3 mmol/l glucose (G3) or 20 mmol/l glucose (G20), with or without 500 µmol/l palmitate and 500 µmol/l ACA (HELSS was not present during the incubation). Because the concentration of BSA was 80 µmol/l (to achieve molar ratio palmitate:BSA = 6.25), the concentration of ACA was increased fivefold as compared with Fig. 7. After 60 min of incubation, an aliquot of the medium was taken for insulin assay. Values are means ± SE for 15 batches of islets from three separate experiments.

We previously suggested that a rise in the ATP:ADP ratio might underlie or at least contribute to the K^+-ATP channel-independent effect of glucose (13,20). Formycin A is an adenosine analog that is converted to formycin A 5′-triphosphate in isolated islets and increases insulin secretion in the presence of glucose. The mechanisms of this effect have not been elucidated, but they do not involve changes in glucose metabolism or activation of PKA and PKC (60). Addition of

n the presence of high K^+ (Fig. 9). In conclusion, the K^+-ATP hannel–independent effect of glucose on insulin secretion oes not involve the NO pathway or protein kinase G.

Evaluation of various potential mechanisms. Glucose nfluences fluxes and concentration of inorganic phosphate n islet cells (55). However, addition of 1 mmol/l Na_2HPO_4 to ur usual phosphate-free medium did not influence the K^+-ATP channel–independent effect of glucose (Table 2). Glucose timulates Mg^{2+} efflux and net uptake in islet cells (56), but mission of $MgCl_2$ from the medium did not impair the ncrease in K^+-induced insulin secretion by glucose (Table 2).

It is unclear whether phosphatidylinositol 3-kinase partic-pates in the acute control of insulin secretion by glucose 57,58). Two inhibitors of the enzyme, wortmannin and Y294002, did not interfere with the K^+-ATP channel–inde-endent effect of glucose (Table 2).

The heterotrimeric GTP-binding proteins G_i and G_o, which ubserve the inhibition of insulin secretion by a variety of hor-nones and neurotransmitters, can be inactivated by PTX 59). Culture of the islets with PTX antagonized the inhibition f insulin secretion caused by epinephrine (data not shown) ut did not alter the ability of glucose to stimulate insulin ecretion in the presence of high K^+.

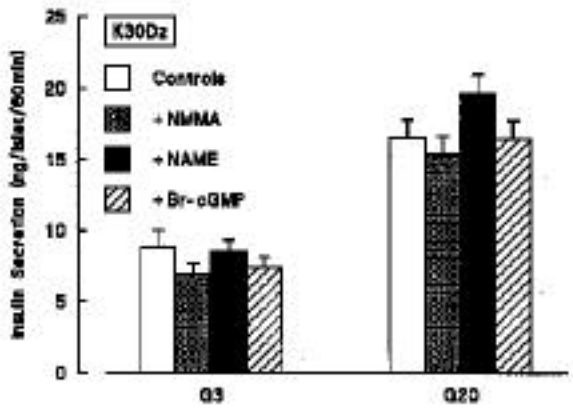


FIG. 9. Influence of NO synthase inhibitors and cGMP on the K^+-ATP channel–independent effect of glucose on insulin secretion. Islets were first preincubated for 60 min in a control medium containing 15 mmol/l glucose. They were then distributed in batches of three in 1 ml of medium containing 30 mmol/l K^+, 250 µmol/l diazoxide, and either 3 mmol/l glucose (G3) or 20 mmol/l glucose (G20). As indicated, the medium was also supplemented with 0.5 mmol/l L-NMMA, 5 mmol/l L-NAME, or 1 mmol/l Br-cGMP. After 60 min of incubation, an aliquot of the medium was taken for insulin assay. Values are means ± SE for 20 batches of islets from four separate experiments.

TABLE 2
Influence of various agents on the K^+-ATP channel–independent effect of glucose on insulin release

Test agent	Insulin secretion (ng · islet $^{-1}$· 60 min^{-1})			
	Glucose (3 mmol/l)		Glucose (20 mmol/l)	
	Controls	+ Test agent	Controls	+ Test agent
HPO_4^{2-} (1 mmol/l)	4.9 ± 0.3	4.6 ± 0.3	10.8 ± 0.7	12.0 ± 0.8
Mg^{2+} (0)	4.9 ± 0.4	6.8 ± 0.5*	12.4 ± 0.7	15.4 ± 0.8*
PTX (200 ng/ml)	3.3 ± 0.2	2.5 ± 0.1	6.3 ± 0.5	5.4 ± 0.3
Wortmannin (10 nmol/l)	4.0 ± 0.3	5.1 ± 0.3	10.2 ± 0.5	13.1 ± 0.8*
LY 294002 (5 µmol/l)	—	3.3 ± 0.2	—	13.4 ± 0.8*

Data are means ± SE for 10–25 batches of islets. After preincubation for 60 min in a control medium containing 3 mmol/l glucose, batches of three islets were incubated in a medium containing 30 mmol/l K^+, 250 µmol/l diazoxide, and the indicated concentration of glucose. The test agents were addition of Na_2HPO_4, omission of $MgCl_2$, and addition of wortmannin or LY294002, as indicated. When the effects of PTX were tested, the islets were cultured for 42 h in RPMI medium containing or not containing 200 ng/ml PTX. *P < 0.05 vs. controls.

0.5 mmol/l formycin A to the incubation medium almost doubled insulin secretion induced by high K^+ in the presence of 10 and 20 mmol/l glucose, but it was without significant effect in the presence of 0 or 3 mmol/l glucose (Fig. 10). However, insulin secretion was strongly potentiated under the latter conditions when formycin A was also added to the preincubation medium (Fig. 10). Formycin A thus appears to reproduce the K^+-ATP channel–independent action of glucose, but the persistence of an effect in the presence of 20 mmol/l glucose indicates that more than one mechanism may be implicated. The present findings and conclusion are in agreement with the observation, made by capacitance measurements, that formycin A increases the efficacy of Ca^{2+} on exocytosis of insulin granules in voltage-clamped mouse β-cells (61), as do glucose and ATP (62).

Conclusions. That a K^+-ATP channel–independent pathway participates in the regulation of insulin secretion by glucose is not disputed. Identification of the underlying mechanism is thus necessary to complete our understanding of the β-cell physiology, to identify possible sites of dysfunction in β-cells from NIDDM patients, and hence to optimize our therapeutic arsenal. We previously reported that a good correlation exists between islet adenine and guanine nucleotides and glucose-induced insulin release via this K^+-ATP channel–independent pathway (13,20). However, such a correlative evidence did not exclude the possibility that the true regulator was different. We, therefore, systematically evaluated the potential role of a number of other mediators, some of which, like long-chain acyl CoAs and arachidonic acid, were regarded as very plausible candidates. None of them appears to be involved. The observation that succinate stimulates exocytosis in permeabilized insulin-secreting cells in which [Ca^{2+}] and [ATP] are buffered prompted the suggestion that mitochondria generate a factor capable of increasing insulin secretion (63). As the nature of this putative factor is still unidentified, its possible participation in the regulatory pathway studied here could not be evaluated. At this stage, we are thus left with our proposal (13,20) that nucleotides may play a dual role in stimulus-secretion coupling: a regulation of the production of the triggering signal, a rise in cytoplasmic $[Ca^{2+}]_i$, through their action on K^+-ATP channels, and a modulation of the action of Ca^{2+} on exocytosis. An action of ATP at this second site has received further support recently (62). If our conclusion eventually turned out to be incorrect, it is likely that serendipity rather than a further systematic approach will provide the final answer to the search for the mechanisms of the K^+-ATP channel–independent pathway of regulation of insulin secretion by glucose.

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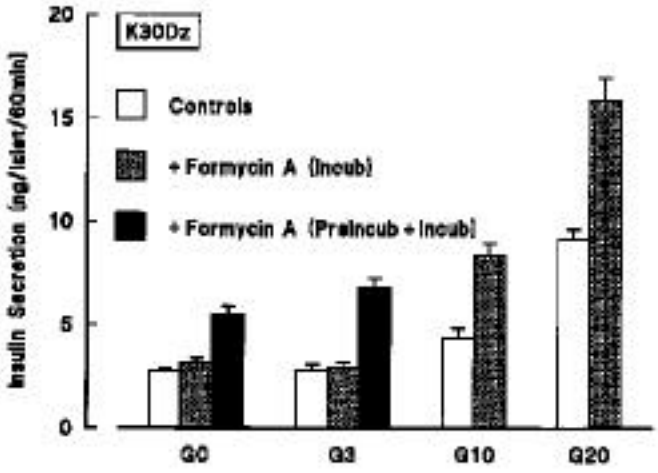

FIG. 10. Influence of formycin A, an adenosine analog, on the K^+-ATP channel–independent effect of glucose on insulin secretion. Islets were first preincubated for 60 min in a control medium containing 3 mmol/l glucose alone or with 0.5 mmol/l formycin A. They were then distributed in batches of three in 1 ml of medium containing 30 mmol/l K^+, 250 µmol/l diazoxide, and the indicated concentration of glucose (G). Formycin A was present in test solutions. After 60 min of incubation, an aliquot of the medium was taken for insulin assay. Values are means ± SE for 10 batches of islets from two separate experiments.

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Author Queries (please see Q in margin and underlined text)

OK to add “In” for subtitle?

Table 1: OK to add “mmol/l” ?

Q1: ATP instead of AVP correct?

Q2: **‘...of the islet content’ correct? or ‘in the islet content’ meant?**

Q3: ADA style breaks up RESULTS and DISCUSSION. OK where I have inserted DISCUSSION head?