Triggers of Insulin Release by a Combination of cAMP Signal and Nutrients
An ATP-Sensitive K⁺ Channel–Independent Phenomenon
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Nutrient augmentation of Ca²⁺-triggered insulin release occurs in an ATP-sensitive K⁺ (K<sub>ATP</sub>) channel-independent manner. Here, using rat islets, we explored the possibility of the K<sub>ATP</sub> channel-independent nutrient triggering of insulin release. In the presence of 250 μmol/l diazoxide, simultaneous application of forskolin and 16.7 mmol/l glucose strongly stimulated insulin release: fourfold and eightfold increases with 1 and 30 μmol/l forskolin, respectively. α-Ketoisocaproate (KIC) and 3-isobutylmethylxanthine (IBMX) could be used in place of glucose and forskolin, respectively, to trigger insulin release in the presence of diazoxide. Triggering of insulin release by a combination of nutrients and forskolin was not attenuated by 10 μmol/l nifedipine (a blocker of voltage-dependent Ca²⁺ channels) and 2 μmol/l thapsigargin (an inhibitor of intracellular Ca²⁺-ATPase), ascertaining independence of this phenomenon from Ca²⁺ entry and from intracellular Ca²⁺ liberation. As anticipated, the action of glucose and KIC was greatly (>80%) suppressed by inhibition of mitochondrial metabolism by 2 mmol/l sodium azide (Na<sub>N</sub>₃). A combination of palmitate and dimethyl glutamate (a cell-permeable glutamate donor), but not either one alone, weakly but unequivocally triggered insulin release when applied simultaneously with forskolin. In this case, however, mitochondrial poisoning by azide was without effect. The finding suggests that a combination of induced palmitoylation and cytosolic glutamate accumulation partially reconstituted signaling beyond mitochondrial metabolism in the β-cell upon glucose stimulation. In conclusion, a combination of cAMP signal and nutrients potently triggers insulin release under full activation of the K<sub>ATP</sub> channel, indicating the multiplicity of driving force for insulin exocytosis. Diabetes 51 (Suppl. 1):S29–S32, 2002

It has been well established that glucose stimulates insulin release by the pancreatic β-cell through ATP-sensitive K⁺ channel (K<sub>ATP</sub> channel)-dependent and -independent signaling pathways (1–3). Accordingly, a biphasic insulin release in response to a square-wave application of high glucose is usually envisaged as follows. Glucose initially triggers insulin release through the K<sub>ATP</sub> channel-dependent, ionic events leading to elevation of submembrane free Ca²⁺ concentration, which produces the first phase. Subsequently, the Ca²⁺-triggered insulin release is gradually augmented by the K<sub>ATP</sub> channel-independent, nonionic signals, the nature of which is still only vaguely understood. The second phase is thus formed (4). Here, the K<sub>ATP</sub> channel-dependent events are regarded as indispensable for initiation of insulin secretion, and the K<sub>ATP</sub> channel-independent glucose actions are considered ancillary to the K<sub>ATP</sub> channel-dependent events.

However, glucose regulation of insulin secretion is clearly present in the patients without functional β-cell K<sub>ATP</sub> channels (5) and also in vitro in the islets isolated from such patients (6). Moreover, in the mouse with targeted disruption of the genes encoding either of the subunits of the K<sub>ATP</sub> channel, Kir6.2, or SUR1, glucose intolerance never develops under regular feeding (7,8). Thus, glucose regulates or, more correctly, triggers insulin release independently of its action on the K<sub>ATP</sub> channels. In the present study, we explored such a possibility using freshly isolated rat pancreatic islets in vitro.

RESEARCH DESIGN AND METHODS
Isolation of pancreatic islets. Male Wistar rats weighing 250–450 g were killed by CO₂ asphyxiation. Immediately after death, the pancreases were surgically removed and the islets were isolated by collagenase digestion (9). Krebs-Ringer bicarbonate (KRB) buffer containing 129 mmol/l NaCl, 5 mmol/l NaHCO<sub>3</sub>, 4.8 mmol/l KCl, 1.2 mmol/l KH<sub>2</sub>PO<sub>4</sub>, 1.2 mmol/l MgSO<sub>4</sub>, 2.5 mmol/l CaCl<sub>2</sub>, 5.6 mmol/l glucose, 0.1% bovine serum albumin (BSA), and 10 mmol/l HEPES at pH 7.4 was used for isolation and pooling of the islets. Measurements of insulin release. Insulin release was measured in static incubation experiments at 37°C using KRB buffer containing 0.68% (100 μmol/l) fatty acid–free BSA (Sigma, St. Louis, Mo). Batches of five size-matched islets/tube were used. The islets were first incubated in 1 ml KRB buffer containing 2.8 mmol/l glucose and 250 μmol/l diazoxide for 30 min (preincubation). After the preincubation, the buffer was removed by aspiration, 1 ml of fresh KRB buffer with one or more test substances was introduced, and the incubation was continued for 60 min (test incubation). When palmitate was used, it was dissolved at a concentration of 600 μmol/l in 1 ml of KRB buffer.
KRB buffer containing 0.68% (100 μmol/l) fatty acid-free BSA. In the presence of 100 μmol/l BSA, 600 μmol/l palmitate gives an estimated free palmitate concentration of 10 μmol/l (10). Nifedipine or thapsigargin (Sigma) was present throughout the preincubation and incubation periods when indicated. Forskolin, α-ketocapric acid (KIC), palmitate, 3-isobutylmethylxanthine (IBMX), and glucagon-like peptide (GLP)-1(7–37) amide were purchased from Sigma. l-Glutamic acid dimethyl ester hydrochloride (dimethyl glutamate [DMG]) was obtained from Fluka Chemie. The same final concentrations of DMG were present in paired control tubes. The pH of test solutions was adjusted to 7.4 immediately before use. At the end of test incubation, the medium was aspirated and kept at 20°C until radioimmunoassay for insulin, where rat insulin was used as standard.

RESULTS

**K<sub>ATP</sub> channel-independent action of nutrients on Ca<sup>2+</sup>-stimulated insulin release.** We examined insulin release from rat pancreatic islets in the presence of 250 μmol/l diazoxide, an opener of K<sub>ATP</sub> channels, to dissect out the K<sub>ATP</sub> channel-independent insulinothrow action. As shown in Fig. 1A, if tested alone, 16.7 mmol/l glucose, 20 mmol/l KIC, 10 μmol/l palmitate, and 10 μmol/l DMG did not stimulate insulin release in the presence of diazoxide. However, when the β-cell was depolarized and [Ca<sup>2+</sup>]<sub>i</sub> was raised by a high concentration of K<sup>+</sup>, 16.7 mmol/l glucose and 20 mmol/l KIC markedly augmented insulin release, as previously reported (11,12). In contrast, 10 μmol/l palmitate did not augment Ca<sup>2+</sup>-stimulated insulin release (Fig. 1A). DMG has been used as a cell-permeable glutamate donor (12,13), and glutamate was recently proposed as a conveyor of K<sub>ATP</sub> channel-independent glucose action (13). Nevertheless, application of 10 mmol/l DMG together with a depolarizing concentration of K<sup>+</sup> exhibited only a tiny augmentation of Ca<sup>2+</sup>-stimulated insulin release, as reported (14). A combination of palmitate and DMG also failed to cause significant augmentation of Ca<sup>2+</sup>-stimulated insulin release.

**Effects of forskolin or a combination of the nutrients and forskolin in the presence of diazoxide.** To explore a possible interaction of cAMP signaling and the K<sub>ATP</sub> channel-independent nutrient actions, we next examined effects of a combination of the nutrients and forskolin on insulin release in the presence of diazoxide. As shown in Fig. 1B, 1 and 30 μmol/l forskolin alone marginally stimulated insulin release in the presence of diazoxide. When 16.7 mmol/l glucose was applied simultaneously with forskolin, a fourfold (with 1 μmol/l forskolin) and eightfold (with 30 μmol/l forskolin) increase in insulin release occurred. A combination of KIC (20 mmol/l) and forskolin also stimulated insulin release (a fivefold increase). The results indicate that glucose and KIC stimulate insulin release independently of its action on the K<sub>ATP</sub> channel without forced depolarization, provided cAMP is increased. In contrast, palmitate (10 μmol/l) or DMG (10 mmol/l) did not stimulate insulin release even when they were applied simultaneously with 1 μmol/l forskolin. Interestingly, however, when the islets were stimulated with a combination of palmitate, DMG, and forskolin, there was a twofold increase in insulin release (Fig. 1B, the far-right bar in the righthand panel). Addition of formycin (0.5 mmol/l), a cell-permeable ATP analog (15), on top of this combination, did not further augment insulin release (data not shown).

**Effects of nifedipine or thapsigargin on insulin release induced by a combination of the nutrients and forskolin in the presence of diazoxide.** Even in the presence of 250 μmol/l diazoxide, it is theoretically possible that a combination of nutrients and forskolin increase [Ca<sup>2+</sup>]<sub>i</sub> because of direct activation of the voltage-dependent calcium channels (VDCC) or mobilization of Ca<sup>2+</sup> from the intracellular Ca<sup>2+</sup> store. We therefore examined the effect of nifedipine, a blocker of VDCC, and thapsigargin, an inhibitor of intracellular Ca<sup>2+</sup>-ATPase. As shown in Fig. 2, nifedipine at 10 μmol/l, which is sufficient to fully block VDCC of the β-cell (16), had no effect on the insulin release triggered by a combination of the nutrients and forskolin (Fig. 2, middle bars). When the same stimulation was applied in the presence of 2 μmol/l thapsigargin, which almost entirely depletes the intracellular Ca<sup>2+</sup> store (17), 16.7 mmol/l glucose and 20 mmol/l KIC produced a larger insulin response than in the absence of thapsigargin. Insulin release in response to a combination of palmitate, DMG, and forskolin was slightly suppressed by nifedipine, but not affected by thapsigargin (Fig. 2).

**Effects of sodium azide on insulin release induced by a combination of the nutrients and forskolin.** In Fig. 3 are shown the effects of metabolic inhibition by 2 mmol/l sodium azide (NaN<sub>3</sub>) a mitochondrial poison, on insulin release. NaN<sub>3</sub> had no effect on basal insulin release. NaN<sub>3</sub> at 2 mmol/l strongly inhibited insulinothrow action of 16.7 mmol/l glucose (by 88%) and 20 mmol/l KIC (by 82%) applied simultaneously with 1 μmol/l forskolin. In contrast, insulin release induced by a combination of 10 μmol/l palmitate, 10 mmol/l DMG, and 1 μmol/l forskolin...
was not at all attenuated by 2 mmol/l NaN₃. The results indicate that synergism of the cAMP signal and palmitate and DMG does not require mitochondrial metabolism.

**Effects of a combination of GLP-1/IBMX and glucose in the presence of diazoxide.** Figure 4 shows effects of IBMX and a combination of IBMX and GLP-1 (100 nmol/l) on insulin release in the presence of 250 μmol/l diazoxide. In the absence of these agents, 16.7 mmol/l glucose did not stimulate insulin release. A combination of GLP-1 and 16.7 mmol/l glucose did not stimulate insulin release. However, a combination of IBMX and glucose caused significant insulin release, which was clearly dependent on the concentration of IBMX. When GLP-1 was tested in the presence of IBMX, it further augmented insulin release (Fig. 4). IBMX alone had a minimum stimulatory effect both at 0.1 and 1 mmol/l.

**DISCUSSION**

A distinction between triggering (or initiation) of insulin release and augmentation (or potentiation/amplification) of triggered release was first proposed some 30 years ago by Grodsky (18) and has been well accepted ever since (19). In the case of glucose, closure of the KATP channel and resultant elevation of [Ca²⁺]ᵢ is generally considered to be responsible for the triggering of insulin release (19). On the other hand, nonfuel secretagogues, which increase cellular cAMP, have repeatedly been shown not to trigger insulin release but to augment insulin release triggered by elevation of [Ca²⁺]ᵢ (20–24). Nonetheless, here we demonstrated that a combination of cAMP and KATP channel-independent nutrient signals strongly trigger insulin release by the islet β-cell. The insulinotropic effect of a combination of the nutrients and forskolin seen here was not obliterated by blockade of the L-type VDCC and depletion of the intracellular Ca²⁺ store. The finding strongly indicates that elevation of [Ca²⁺]ᵢ due to Ca²⁺ influx through the L-type VDCC or liberation of Ca²⁺ from the intracellular store site is not involved. It should be noted that a combination of forskolin and a high concentration of glucose did not trigger insulin release when the experiments were performed in a buffer without added Ca²⁺-containing EGTA and using islets preexposed to these stringently Ca²⁺-free conditions (25,26). Therefore, not an elevation of [Ca²⁺]ᵢ, but a basal (resting) level of it appears to be needed for the triggering of insulin release by the combination of cAMP and nutrient signals.

In the case of glucose and KIC, mitochondrial metabolism was critically required for this insulinotropic action. However, when a combination of palmitate (a putative inducer of palmitoylation) and DMG (a cell-permeable glutamate donor) was employed in place of glucose, insulin release in the presence of forskolin was not at all inhibited by mitochondrial poisoning. Contrary to our expectations, the insulinotropic effect of a combination of
palmitate and DMG was much weaker than the maximum glucose effect, and an addition of formycin A on top of the two did not further stimulate insulin release. Thus, we consider a combination of induced palmitoylation and cytosolic accumulation of glutamate partially reconstituted postmitochondrial events going on in the β-cell upon glucose stimulation.

IBMX could be used in the place of forskolin to let the nutrients trigger insulin release in a K\textsubscript{ATP} channel-independent manner. Although a combination of GLP-1 and glucose failed to trigger insulin release in the presence of diazoxide, GLP-1 further augmented insulin release, provided phosphodiesterase was inhibited by IBMX. Therefore, the cAMP signal acts in synergy with the K\textsubscript{ATP} channel-independent nutrient action. Because stimulation of the β-cell by incretins such as GLP-1 becomes stronger postprandially, the mechanism shown here may well be contributing to a postprandial, rapid increase of insulin secretion from the β-cell. Maintenance of life-long euglycemia in mice with targeted disruption of the genes encoding either subunit of the K\textsubscript{ATP} channel (7,8) can be explained based on the current findings. Nishimura et al. (27) previously reported a similar phenomenon using a different experimental protocol.

Concentrations of 1 and 30 μmol/l forskolin and 0.1 and 1 mmol/l IBMX increase cellular cAMP in a concentration-dependent manner (24,25). However, insulin release elicited by these agents in the absence of glucose was only marginal and not concentration-dependent. When the K\textsubscript{ATP} channel-independent nutrient signal was added on top of cAMP, insulin release was clearly dependent on the concentration of forskolin or IBMX. The fact suggests that the synergism between cAMP and nonionic nutrient action is required for operation of the tuning of insulin release by cAMP.

In conclusion, we tried a simultaneous activation of the signals that have been considered not to trigger (or initiate) insulin release. This procedure in fact strongly triggered insulin release. This finding provides insight into the β-cell stimulus–secretion coupling in vivo where not one but many signals are simultaneously activated, especially after meals.

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