Activation of the $K_{ATP}$ channel-independent signaling pathway by the nonhydrolyzable analog of leucine, BCH

Yi-Jia Liu,1 Haiying Cheng,1 Heather Drought,2 Michael J. MacDonald,2 Geoffrey W. G. Sharp,1 and Susanne G. Straub1

1Department of Molecular Medicine, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853; and 2University of Wisconsin Children’s Diabetes Center, Madison, Wisconsin 53706

Submitted 7 January 2003; accepted in final form 18 April 2003

Liu, Yi-Jia, Haiying Cheng, Heather Drought, Michael J. MacDonald, Geoffrey W. G. Sharp, and Susanne G. Straub. Activation of the $K_{ATP}$ channel-independent signaling pathway by the nonhydrolyzable analog of leucine, BCH. Am J Physiol Endocrinol Metab 285: E380–E389, 2003.—Leucine and glutamine were used to elicit biphasic insulin release in rat pancreatic islets. Leucine did not mimic the full biphasic response of glucose. Glutamine was without effect. However, the combination of the two did mimic the biphasic response. When the ATP-sensitive K+ ($K_{ATP}$) channel-independent pathway was studied in the presence of diazoxide and KCl, leucine and its nonmetabolizable analog 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH) both stimulated insulin secretion to a greater extent than glucose. Glutamine and dimethyl glutamate had no effect. Because the only known action of BCH is stimulation of glutamate dehydrogenase, this is sufficient to develop the full effect of the $K_{ATP}$ channel-independent pathway. Glucose, leucine, and BCH had no effect on intracellular citrate levels. Leucine and BCH both decreased glutamate levels, whereas glucose was without effect. Glucose and leucine decreased palmitate oxidation and increased esterification. Strikingly, BCH had no effect on palmitate oxidation or esterification. Thus BCH activates the $K_{ATP}$ channel-independent pathway of glucose signaling without raising citrate levels, without decreasing fatty acid oxidation, and without mimicking the effects of glucose and leucine on esterification. The results indicate that increased flux through the TCA cycle is sufficient to activate the $K_{ATP}$ channel-independent pathway.

ATP-sensitive K+ channel; 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid

AT LEAST TWO MAJOR SIGNALING PATHWAYS are involved in glucose-stimulus secretion coupling. These are the ATP-sensitive K+ ($K_{ATP}$) channel-dependent and $K_{ATP}$ channel-independent pathways. The former is responsible for the first phase of glucose-stimulated insulin secretion, and the two pathways combined are responsible for the second phase of glucose-stimulated insulin secretion (52, 54). The $K_{ATP}$ channel-dependent pathway closes $K_{ATP}$ channels (4, 13). This results in depolarization, $Ca^{2+}$ entry via voltage-gated $Ca^{2+}$ channels, raised cytosolic $Ca^{2+}$ concentration ([Ca$^{2+}$]), and the stimulation of secretion. The $K_{ATP}$ channel-independent pathway (6, 19, 48) works in synergy with the $K_{ATP}$ channel-dependent pathway because of a requirement for increased [Ca$^{2+}$]. It should be noted, however, that simultaneous activation of PKA and PKC circumvents the Ca$^{2+}$ requirement (28–30). Glucose and other nutrients activate the pathway (6, 18, 19, 29, 48), and nutrient-driven anaplerosis is regarded as essential for its operation (10, 17). Whereas the underlying mechanisms involved in the $K_{ATP}$ channel-dependent pathway are well defined (1, 4, 13, 23), the mechanisms involved in the $K_{ATP}$ channel-independent pathway are not yet known and are subject to considerable controversy.

Four present hypotheses regarding the $K_{ATP}$ channel-independent pathway are as follows. 1) As a result of anaplerosis, glucose induces an increase in mitochondrial citrate, cytosolic citrate, and cytosolic malonyl-CoA. The resulting malonyl-CoA-induced inhibition of carnitine palmitoyltransferase (CPT)-1 and diversion of fatty acids away from oxidation result in the generation of increased amounts of long-chain acyl-CoA and other potential signaling molecules in the cytosol (10, 14, 44). 2) Glucose induces an increase in the cytosol, perhaps by an action on the granules (38). 3) Changes in adenine nucleotide concentrations are regulators of the $K_{ATP}$ channel-independent pathway (49). 4) Protein acylation is involved (53, 56).

In the present study, we reexamined the idea that only nutrients can activate the $K_{ATP}$ channel-independent pathway and that anaplerosis is essential to it by studying the effects of glucose, leucine, 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH), which is a nonmetabolizable analog of leucine, and glutamine. These agents were studied alone and in combination. Leucine is the most potent amino acid in stimulating insulin release and is thought to be so because of its ability to activate glutamate dehydrogenase (GDH) in addition to its metabolism as a fuel (20, 27, 50). BCH is a selective activator of GDH, and this appears to be the

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
sole mechanism by which it stimulates insulin secretion (16, 20, 27, 39, 43, 50). The provision of leucine and glutamine mimics the pyruvate dehydrogenase and pyruvate carboxylase pathways, respectively, of glucose metabolism by providing mitochondrial acetyl-CoA and ATP, and mitochondrial intermediates (anaplerosis), respectively (Fig. 1). The combination of these two compounds reproduced the effect of glucose to activate both the KATP channel-dependent and KATP channel-independent pathways. Most importantly, it was found that the nonnutrient BCH alone (without leucine) was sufficient to replicate the effect of glucose on the KATP channel-independent pathway of β-cell signaling, and it did so without any suppression of fatty acid oxidation or overall increase in esterification. Our data identify signals from the tricarboxylic acid cycle as key activators of the KATP channel-independent pathway.

MATERIALS AND METHODS

Materials. Glucose, leucine, glutamine, BCH, L-carnitine, and diazoxide were obtained from Sigma (St. Louis, MO). [1-14C]palmitate and 253H-labeled insulin were obtained from New England Nuclear Life Science Products (Boston, MA).

Isolation of pancreatic islets. Male Sprague-Dawley rats (250–350 g) were used in this study and had access to unlimited food and water. After CO2 asphyxiation, the pancreas was surgically removed, and the islets were isolated by collagenase digestion (31).

Insulin secretion under perifusion conditions. Krebs-Ringer bicarbonate-HEPES buffer [KRBH; containing (in mM) 129 NaCl, 5 NaHCO3, 1.2 KH2PO4, 2.5 CaCl2, 1.2 MgSO4, 10 HEPES at pH 7.4, and 0.1% bovine serum albumin (BSA)] was used for the insulin secretion studies. The perifusion protocol was slightly modified from that originally described (26). Briefly, 20 islets were placed into 70-μl perifusion chambers. An equilibration period of 40–45 min with KRBH and 2.8 mM glucose at 37°C was followed by the test period. Samples were collected at 1- or 2-min intervals at a flow rate of 1 ml/min. Insulin was measured by RIA using a charcoal separation method (21). The nutrient and BCH concentrations in the various experiments are specified in the figure legends and the term basal glucose refers to a concentration of 2.8 mM glucose. When the KATP channel-independent pathway was studied, 0.3 M diazoxide and 40 mM KCl were added simultaneously with the test agents.

Insulin secretion under static incubation conditions. Batches of five size-matched islets per tube were used. The islets were preincubated in KRBH buffer for 60 min and then incubated in the presence and absence of the test agents for 60 min. At the end of the incubations, samples were taken and kept at −20°C for RIA.

Measurement of palmitate incorporation into lipids. The method was similar to that used previously (12). Rat pancreatic islets were preincubated in KRBH buffer containing 2.8 mM glucose for 60 min. Groups of 60–100 islets were then incubated with 200 μl of KRBH containing 0.8 mM L-carnitine, 0.2 μCi [1-14C]palmitate, and the test compounds (glucose, leucine, diazoxide, and KCl) in Eppendorf tubes in a 37°C incubator for 60 min. At the end of the incubation, the media were removed and the islets washed twice with 0.3 ml of ice-cold PBS. Immediately following, 200 μl of 0.2 M NaOH was added, and the samples were vortexed vigorously for 2 min. After centrifugation at 2,000 g, 0.5 ml of the bottom lipid-soluble layer was washed twice with 200 μl of chloroform-methanol (2:1) and 50 μl of 0.1 N KOH were added and the samples were incubated at 37°C for 2 h. After washing, the samples were washed twice with 200 μl of ice-cold PBS. Immediately after addition of 200 μl of 0.2 M NaCl, the islet pellets were frozen in liquid N2. After thawing, 750 μl of chloroform-isoamyl alcohol (24:1) and 50 μl of 0.1 N HCl were added, and the samples were vortexed vigorously for 2 min. After centrifugation at 2,000 g, 0.5 ml of the upper aqueous phase was washed twice with 200 μl of chloroform-methanol (2:1). The aqueous phase was then passed through a 0.45-μm syringe filter and collected. The islet pellets were washed twice with 200 μl of CHCl3, and the aqueous phase was withdrawn. The aqueous phase was filtered into scintillation counting vials and solubilized by the addition of 1 ml of scintillation cocktail.after which the samples were analyzed for 14C radioactivity by liquid scintillation spectrometry.

Measurement of palmitate oxidation. The method was similar to that used previously (12). Rat pancreatic islets were preincubated in KRBH buffer containing 2.8 mM glucose for 60 min. Groups of 60–100 islets were then incubated with 200 μl of KRBH containing 0.8 mM L-carnitine, 0.2 μCi [1-14C]palmitate, and the test compounds (glucose, leucine, diazoxide, and KCl) in Eppendorf tubes for 37°C incubator for 60 min. At the conclusion of the incubation, the media were removed and the islets washed twice with 0.3 ml of ice-cold PBS. Immediately after addition of 200 μl of 0.2 M NaCl, the islet pellets were frozen in liquid N2. After thawing, 750 μl of chloroform-methanol (24:1) and 50 μl of 0.1 N KOH were added, and the samples were vortexed vigorously for 2 min. After centrifugation at 2,000 g, 0.5 ml of the upper aqueous phase was washed twice with 200 μl of chloroform-methanol (24:1). The aqueous phase was then passed through a 0.45-μm syringe filter and collected. The islet pellets were washed twice with 200 μl of CHCl3, and the aqueous phase was withdrawn. The aqueous phase was filtered into scintillation counting vials and solubilized by the addition of 1 ml of scintillation cocktail. After which the samples were analyzed for 14C radioactivity by liquid scintillation spectrometry.
E382

BCH ACTIVATES K<sub>ATP</sub> CHANNEL-INDEPENDENT PATHWAY

L-carnitine and 0.2 μCi [1-<sup>14</sup>C]palmitate, as described above. After two washes in PBS, 200 μl of chloroform and 100 μl of methanol-HCl (100:1) were added and the samples stored overnight at 4°C. The following day, 75 μl of double-distilled H<sub>2</sub>O were added to each of the samples, and after vigorous vortexing and a 10-min centrifugation at 4°C the upper aqueous phase was removed. This step was repeated once, and the organic (lower) phase was dried down under liquid N<sub>2</sub>. Dried-down samples were reconstituted in a mixture of chloroform-methanol (1:1), and a lipid carrier mixture was added to each of the samples consisting of tri-, di-, and monoacylglycerols, phospholipids, and palmitic acid. After separation by thin-layer chromatography [solvent system: n-hexane-diethyl ether-methanol-acetic acid (90:20:2:3)], the plates were sprayed with 2′,7′-dichlorofluorescein, and single spots were visualized under UV light. Individual spots were cut out, transferred to glass vials, and after addition of 0.5 ml of methanol and 5 ml of BioSafe<sup>2</sup> scintillation fluid, radioactivity was determined by liquid scintillation counting.

Metabolite assays. Batches of 100 rat islets were incubated for 30 min at 37°C in KRBH in the presence and absence of the various insulin secretagogues used for the insulin secretion studies. Metabolites were measured by alkali-enhanced fluorescence of NAD(P)(H), as previously described (37).

Statistical analysis. All data were subjected to one-way ANOVA.

RESULTS

In Fig. 2A are shown the effects of 16.7 mM glucose, 20 mM leucine, and 10 mM glutamine alone, and the combination of leucine plus glutamine, on insulin secretion under paired perifusion conditions. Glucose-stimulated biphasic insulin release with a peak first-phase response 5 min after exposure to glucose. A nadir after 8 min was followed by a rising second phase, which reached a plateau after 20–30 min. The response to 20 mM leucine was similar but with two important distinctions. The first phase of the response occurred slightly earlier, likely due to the more rapid metabolism of leucine relative to that of glucose, and the amount of insulin released during the second phase was significantly less than that caused by glucose. Glutamine was without effect on secretion, whereas the combination of glutamine and leucine precisely mimicked the second phase of glucose-stimulated insulin secretion. As anticipated (8, 45), when dimethyl glutamate was used in place of glutamine, similar results were obtained (data not shown). Dimethyl glutamate alone had no effect on insulin secretion but potentiated the effect of leucine.

To distinguish between the effects of leucine metabolism and the activation of GDH in the generation of the responses, similar paired experiments were performed using BCH instead of leucine. The results are shown in Fig. 2B. Glucose and glutamine gave similar data to those in Fig. 2A. Interestingly, BCH reproduced the effect of leucine both alone and in combination with glutamine. Glutamine was without effect on secretion, whereas the combination of glutamine and BCH mimicked the second phase of glucose-stimulated insulin.

These data are in accord with the idea that both arms of glucose metabolism, the production of acetyl-CoA and ATP, and the provision of TCA cycle intermediates are required for the full biphasic response to glucose. Additionally, the fact that BCH alone mimicked the effect of leucine suggests that activation of GDH is the dominant effect of leucine and that its metabolism under these conditions is of little importance in stimulus-secretion coupling.

In the next experiments, we examined the effects of glucose, leucine and glutamine, and BCH and glutamate on the K<sub>ATP</sub> channel-independent pathway of β-cell signaling (see Fig. 3). In this paradigm, the first phase of the glucose response is mimicked by the depolarizing action of KCl, whereas the K<sub>ATP</sub> channel-independent pathway provides the second phase. The response to glucose and KCl was slightly biphasic, with a peak response after 5 min, a slight nadir, and a subsequent rising second phase. The responses to leucine plus glutamine and BCH plus glutamine in the presence of KCl and diazoxide were similar to each
Fig. 3. Effects of 16.7 mM glucose and the combinations of 20 mM leucine + 10 mM glutamine, and 20 mM BCH + 10 mM glutamine on insulin secretion from rat islets under the K<sub>ATP</sub> channel-independent paradigm in the presence of 250 μM diazoxide and 40 mM KCl (n = 6). Over the 1st phase, glucose-stimulated insulin secretion averaged 80 ± 4 pg·islet<sup>−1</sup>·min<sup>−1</sup> and leucine + glutamine-stimulated release 125 ± 6 pg·islet<sup>−1</sup>·min<sup>−1</sup>, Δ = 45 ± 9 pg·islet<sup>−1</sup>·min<sup>−1</sup>, P < 0.001, n = 6. BCH + glutamine-stimulated release averaged 128 ± 5 pg·islet<sup>−1</sup>·min<sup>−1</sup>, Δ (vs. glucose) = 48 ± 10 pg·islet<sup>−1</sup>·min<sup>−1</sup>, P < 0.001, n = 6. Over the 2nd phase, glucose-stimulated insulin secretion averaged 126 ± 3 pg·islet<sup>−1</sup>·min<sup>−1</sup> and leucine + glutamine-stimulated release 183 ± 12 pg·islet<sup>−1</sup>·min<sup>−1</sup>, Δ = 57 ± 6 pg·islet<sup>−1</sup>·min<sup>−1</sup>, P = 0.001, n = 6. BCH + glutamine-stimulated release averaged 183 ± 11 pg·islet<sup>−1</sup>·min<sup>−1</sup>, Δ (vs. glucose) = 57 ± 6 pg·islet<sup>−1</sup>·min<sup>−1</sup>, P < 0.01, n = 6.

Fig. 4. Effects of 16.7 mM glucose, 20 mM leucine, 20 mM BCH, and 20 mM glutamine on insulin secretion from rat islets under the K<sub>ATP</sub> channel-independent paradigm in the presence of 250 μM diazoxide and 40 mM KCl (n = 6). Over the first 20 min (minutes 10–30), leucine and BCH alone stimulated insulin secretion via the K<sub>ATP</sub> channel-independent pathway to a significantly greater extent than glucose. Subsequently, the responses to glucose and BCH were similar, whereas the response to leucine was slightly, but not significantly, less than that of glucose.

Although leucine provides acetyl-CoA and activates GDH, the result obtained with BCH demonstrates that activation of GDH alone is sufficient to reproduce the effects of glucose on the K<sub>ATP</sub> channel-independent pathway. Neither glutamine nor dimethyl glutamate (not shown) had any effect on secretion, because the effects of glutamine plus KCl or of dimethyl glutamate plus KCl were the same as those of KCl alone.

The importance of the activation of the TCA cycle can be seen in the response to succinate used as a succinic acid methyl ester to study the effects of mitochondrial metabolism. Succinate stimulated biphasic insulin release comparably to glucose (Fig. 5A) and strongly stimulated the K<sub>ATP</sub> channel-independent pathway in the presence of KCl and diazoxide (Fig. 5B).

We next determined the effects of the various secretagogues on palmitate oxidation. The results of five paired experiments performed in the absence and presence of 40 mM KCl and 250 μM diazoxide are summarized in Fig. 6. In the absence of KCl and diazoxide, palmitate oxidation was reduced 42% by 16.7 mM glucose (P < 0.05) and 27% by 20 mM leucine (P < 0.05). In sharp contrast, 20 mM BCH slightly increased palmitate oxidation (13%, P < 0.05). When the measurements were repeated in the presence of 40 mM KCl and 250 μM diazoxide, the results were essentially the same. Palmitate oxidation was reduced 51% by 16.7 mM glucose (P < 0.01) and 51% by 20 mM leucine (P < 0.01). BCH under these conditions had no effect. The importance of these data is that, of all the compounds tested, BCH failed to decrease palmitate oxidation despite its similar effect on insulin secretion and ability to stimulate the K<sub>ATP</sub> channel-independent pathway. This effect of BCH had been reported previously (39), but its significance to the mechanisms of augmentation could not have been realized at that time.

When glutamine and the combinations of either leucine or BCH plus glutamine were examined, palmitate oxidation was reduced 58% by 10 mM glutamine (P < 0.01), 64% by 20 mM leucine plus 10 mM glu-
tamine \((P < 0.001)\), and 41% by 20 mM BCH plus 10 mM glutamine \((P < 0.05)\). In the presence of 40 mM KCl and 250 \(\mu\)M diazoxide, palmitate oxidation was reduced 61% by glutamine \((P < 0.001)\), 61% by leucine plus glutamine \((P < 0.001)\), and 49% by BCH plus glutamine \((P < 0.01)\). The results are shown in Fig. 6.

The effects of 16.7 mM glucose, 20 mM BCH, and 20 mM leucine on palmitate incorporation into lipids were determined, and the results are presented in Fig. 7. Glucose at 16.7 mM and 20 mM leucine both increased the esterification of palmitate \((P < 0.005\) for both), whereas 20 mM BCH had no effect. Similar results were obtained when the studies were performed in the presence of 40 mM KCl and 250 \(\mu\)M diazoxide (again \(P < 0.005\) for both glucose and leucine).

Additional studies were performed in an attempt to detect small changes that might have been obscured by the method that measures the incorporation of \([^{14}\text{C}]\)palmitate into total lipids. Thus, after the islets were labeled in the presence of \([^{14}\text{C}]\)palmitate and the same stimuli as in Fig. 7 over a 30-min period, lipids were separated by thin-layer chromatography into various lipid subclasses. Glucose significantly increased the incorporation of palmitate into phospholipids \((P < 0.05)\) and triglycerides \((P < 0.05)\) but not into monoacylglycerols or diacylglycerols (DAG). Leucine increased the incorporation into phospholipids \((P < 0.001)\), monoacylglycerols \((P > 0.05)\), and triglycerides \((P < 0.001)\), but not into DAG. BCH had no significant effect on the incorporation of palmitic acid into any of the four subclasses. These data appear to rule out a potential mechanism for the \(K_{\text{ATP}}\) channel-independent pathway.
of GDH and enhanced conversion of glutamate to α-ketoglutarate (37). In line with this, α-ketoglutarate levels were increased by BCH. Because BCH does not stimulate insulin release by its own metabolism, the decrease in glutamate and increase in α-ketoglutarate levels reflect the increased metabolism of endogenous fuel required to supply the TCA cycle, the KAATP channel-independent signaling pathway and subsequent exocytosis. Because diazoxide, which maintains the KAATP channel in the open state, did not prevent the alterations in metabolite levels, the changes induced by BCH were independent of KAATP channel effects. Incubation with 10 mM glutamine, which alone has no effect on insulin secretion, increased glutamate levels eightfold, consistent with previous results (37).

Finally, to address the question whether increased TCA flux alone or long-chain acyl-CoA accumulation alone is sufficient to recapitulate the KAATP channel-independent pathway, the effect of the CPT-1 inhibitor etomoxir was tested alone under the KCl-diazoxide-2.8 mM glucose paradigm, and also in the presence of KCl-diazoxide-2.8 mM glucose plus 3, 10, or 20 mM BCH. In these experiments, 10 μM etomoxir had no effect alone (control 1.03 ± 0.08% of content released vs. etomoxir 1.24 ± 0.12%, n = 11) or in the presence of 3 mM BCH (control 1.92 ± 0.18% of content released/60 min vs. 2.24 ± 0.33%, n = 9), 10 mM BCH (3.72 ± 0.61% of content released/60 min vs. 3.84 ± 0.56%, n = 5), or 20 mM BCH (4.26 ± 0.38% of content released/60 min vs. 5.06 ± 0.70%, n = 5). In contrast, 10 and 20 mM BCH alone significantly augmented insulin release (control 1.03 ± 0.08% of content released/60 min vs. 10 mM BCH 3.72 ± 0.61%, and vs. 20 mM BCH 4.26 ± 0.38%, n = 5; P < 0.001 for both). Thus inhibition of CPT-1 and long-chain acyl-CoA accumulation is insufficient to reproduce the effects of the KAATP channel-independent pathway. In contrast, activation of the TCA cycle is sufficient.

**DISCUSSION**

The biphasic insulin-secretory response to glucose is mimicked by the combination of leucine and glutamine

---

**Table 1. Effect of 20 mM BCH, 20 mM leucine, 40 mM KCl, 250 μM diazoxide, BCH, and leucine in the presence of 250 μM diazoxide and 40 mM KCl, 16.7 mM glucose, and 10 mM glutamine on metabolite levels in isolated rat pancreatic islets**

<table>
<thead>
<tr>
<th>Incubation Conditions</th>
<th>Level of Metabolite in Islets, nmol metabolite/mg islet protein</th>
<th>Malate</th>
<th>Citrate</th>
<th>Glutamate</th>
<th>α-KG</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td></td>
<td>2.1 ± 0.2(6)</td>
<td>2.4 ± 0.6(12)</td>
<td>15 ± 0.9(6)</td>
<td>0.6 ± 0.2(6)</td>
</tr>
<tr>
<td>BCH</td>
<td></td>
<td>2.2 ± 1.0(6)</td>
<td>2.6 ± 0.4(11)</td>
<td>8.8 ± 0.7(7)</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td></td>
<td>2.3 ± 0.2(4)</td>
<td>2.2 ± 1.2(4)</td>
<td>11 ± 0.6(4)†</td>
<td>0.9 ± 0.3(4)</td>
</tr>
<tr>
<td>KCl</td>
<td></td>
<td>2.4 ± 0.4(4)</td>
<td>2.1 ± 0.5(4)</td>
<td>16 ± 0.8(4)</td>
<td>0.9 ± 0.1(4)</td>
</tr>
<tr>
<td>Diazoxide</td>
<td></td>
<td>2.0 ± 0.7(4)</td>
<td>2.4 ± 0.2(4)</td>
<td>20 ± 2.3(4)</td>
<td>0.6 ± 0.2(4)</td>
</tr>
<tr>
<td>BCh, KCl, diazoxide</td>
<td></td>
<td>2.1 ± 0.2(4)</td>
<td>2.2 ± 0.5(13)</td>
<td>7.9 ± 0.7(8)</td>
<td></td>
</tr>
<tr>
<td>Leucine, KCl, diazoxide</td>
<td></td>
<td>2.7 ± 0.4(4)</td>
<td>2.4 ± 0.3(4)</td>
<td>10 ± 0.9(4)</td>
<td>0.9 ± 0.3(4)</td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td>2.7 ± 0.6(6)</td>
<td>2.4 ± 0.7(14)</td>
<td>13 ± 1.7(16)</td>
<td>0.5 ± 0.2(8)</td>
</tr>
<tr>
<td>Glutamine</td>
<td></td>
<td>4.5 ± 0.8(4)*</td>
<td>3.7 ± 1.0(8)</td>
<td>120 ± 17(10)</td>
<td>0.7 ± 0.2(4)</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE, with no. of replicate incubations in parentheses. Control was Krebs-Ringer bicarbonate-HEPES with 2.8 mM glucose. BCH, 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid; α-KG, α-ketoglutarate. *P < 0.05, †P < 0.01, ‡P < 0.001 vs. no addition.
(43, 51). This is in accord with the concept that the response to glucose is due to its metabolism to pyruvate and synthesis of acetyl-CoA and ATP via the action of pyruvate dehydrogenase and to anaplerosis of TCA cycle intermediates via the action of pyruvate carboxylase (25, 33–36). A glucose-induced increase in the ATP-to-ADP ratio leads to increased [Ca\(^{2+}\)]\(_i\) and the first phase of the secretory response. Increased TCA cycle activity produces the signals that synergize with the increased [Ca\(^{2+}\)]\(_i\) and induce the second phase. The fact that the combination of the nonmetabolizable BCH and glutamine caused a similar response to that of leucine and glutamine demonstrates that metabolism of leucine is not necessary for the development of a full biphasic response. Subsequent studies on the K\(_{\text{ATP}}\) channel-independent pathway, “isolated” by the use of diazoxide and a depolarizing concentration of KCl, demonstrated that the combination of glutamine and either leucine or BCH mimicked the effect of glucose. With the K\(_{\text{ATP}}\) channels opened by diazoxide, KCl elevates [Ca\(^{2+}\)]\(_i\) to provide a “first phase” of insulin secretion and also the elevation of [Ca\(^{2+}\)]\(_i\); necessary for synergy with signals from the K\(_{\text{ATP}}\) channel-independent pathway that results in the second phase of secretion. Under these conditions, the combination of glutamine with leucine or BCH provided a stronger stimulation of insulin release via the K\(_{\text{ATP}}\) channel-independent pathway than did glucose. Next, we determined whether the K\(_{\text{ATP}}\) channel-independent pathway under these conditions was being stimulated by glutamine alone or by leucine and BCH activation of GDH alone or whether the combination of glutamine and activated GDH was required. Whereas glutamine was unable to mimic the effect of glucose, leucine did. More importantly, the nonmetabolizable leucine analog BCH not only mimicked but exceeded the effect of glucose. It is clear from the BCH data presented in Fig. 4 that, in the presence of increased [Ca\(^{2+}\)]\(_i\), activation of the TCA cycle by activation of GDH is all that is required for the full expression of the K\(_{\text{ATP}}\) channel-independent pathway of β-cell signaling. Enhanced Ca\(^{2+}\) influx leads not only to a rise in cytosolic Ca\(^{2+}\) but also to a rise in intramitochondrial Ca\(^{2+}\), thereby activating Ca\(^{2+}\)-sensitive enzymes such as isocitrate dehydrogenase and α-ketoglutarate dehydrogenase. Although these enzymes can act in the absence of Ca\(^{2+}\), their activity is markedly enhanced by Ca\(^{2+}\) (40, 41).

These results emphasize the critical role of the mitochondria in generating the signals necessary for stimulation of insulin secretion via the K\(_{\text{ATP}}\) channel-independent pathway. Both anaplerosis and activation of GDH provide α-ketoglutarate and other downstream intermediates. It seems likely that α-ketoglutarate, or possibly some metabolite derived from α-ketoglutarate, gives rise to the signals necessary to activate the K\(_{\text{ATP}}\) channel-independent pathway. In support of this, succinate (used as the methyl ester) also induces biphasic insulin release and strongly stimulates the K\(_{\text{ATP}}\) channel-independent pathway in the presence of KCl and diazoxide. Knowledge that simple activation of GDH by BCH is all that is necessary for the K\(_{\text{ATP}}\) channel-independent pathway should be extremely helpful in the identification of the complete signaling system.

The results presented here appear to be incompatible with two current hypotheses concerning the signaling mechanisms underlying the K\(_{\text{ATP}}\) channel-independent pathway. The anaplerosis/malonyl-CoA/long-chain acyl-CoA hypothesis requires that glucose induces an increase in the production of mitochondrial citrate, cytosolic citrate, and cytosolic malonyl-CoA. The resulting malonyl-CoA-induced inhibition of CPT-1 and diversion of fatty acids away from oxidation in the mitochondria results in the generation of increased amounts of cytosolic long-chain acyl-CoA and other potential signaling molecules (10, 12, 14, 15, 42, 44, 45). Despite the large amount of evidence in favor of this hypothesis since it was first proposed (14), the hypothesis is currently controversial for the following reasons. Expression of malonyl-CoA decarboxylase in INS-1 cells, which decreased malonyl-CoA levels, impaired the ability of glucose to suppress fatty acid oxidation and decreased the incorporation of fatty acid into lipid but failed to affect glucose-stimulated insulin secretion (3, 42). Second, triacsin C, an inhibitor of long-chain acyl-CoA synthetase, reduced fatty acid oxidation and incorporation of glucose into lipids but had no effect on glucose-stimulated insulin release (3). A third reason is that much of the evidence in favor of the hypothesis has been obtained from work on clonal β-cell lines that do not exhibit the K\(_{\text{ATP}}\) channel-independent pathway (22, 42, 52) and that may display different metabolic pathways to those of the native β-cell. With respect to the data in the present study, we could not show an increase in citrate levels in response to glucose or other combinations of agents that stimulate the K\(_{\text{ATP}}\) channel-independent pathway. Furthermore, BCH, which strongly activates this pathway, fails to reduce palmitate oxidation or to increase palmitate incorporation into lipids as does glucose. Although our findings are difficult to reconcile with a hypothesis that involves a rise in citrate and malonyl-CoA and a decrease in fatty acid oxidation, the data provided by the authors of this hypothesis with respect to the need for anaplerosis (10, 14, 44) remain strong and are supported by our data.

A second hypothesis states that glucose induces an increase in the production in the mitochondria of glutamate, which is exported to the cytosolic compartment and sensitizes the secretory machinery to Ca\(^{2+}\) (38). This hypothesis is also controversial (5, 37, 57). Evidence in favor is the report that glucose generated glutamate from β-cell mitochondria, that a membrane-permeant glutamate analog sensitized the glucose-induced secretory response, and that glutamate added directly to permeabilized INS-1 cells increased Ca\(^{2+}\)-stimulated insulin secretion. Note again, however, that the INS-1 cell used does not manifest the K\(_{\text{ATP}}\) channel-independent pathway. In addition, the hypothesis is supported by experiments in which overexpression of glutamate decarboxylase (GAD) in INS-1 cells was used to reduce cytosolic glutamate levels. In these cells, insulin secretion in response to 15 mM glucose...
was reduced by 37% compared with controls, whereas the secretory response to KCl was unaffected. Similar results were obtained in perfused rat pancreatic islets after adenovirus transfection of GAD (47). The evidence against this hypothesis is that we here and others (37) report that glucose does not increase the levels of intracellular glutamate. Also, as has been shown here and by others (5, 37), glutamine raises glutamate levels markedly without stimulating insulin release and without augmenting stimulated insulin release in the presence of KCl and diazoxide. Dimethyl glutamate similarly had no effect on secretion, as already reported (57). However, the fuel effect of both glutamine and dimethyl glutamate can be detected when GDH is activated by leucine or BCH.

Our data do not address the third hypothesis, that changes in adenine and or guanine nucleotide concentrations are regulating the KATP channel-independent pathway (49). However, the activity of the KATP channel-independent pathway was unaltered under conditions in which the ATP-to-ADP ratio was decreased (2). In this study, chronic exposure of islets to leucine resulted in impaired glucose-stimulated insulin secretion and was associated with a decreased ATP-to-ADP ratio relative to control islets. This defect was presumed responsible for the impaired secretion because of the failure of glucose to decrease KATP channel activity and depolarize the β-cell. When the effect of glucose via the KATP channel-independent pathway was studied in the presence of KCl and diazoxide, the response was unaffected and was the same as in control islets. This is the most direct evidence yet available against the hypothesis that adenine nucleotides have a major role in the control of the KATP channel-independent pathway. With respect to guanine nucleotides, depletion of cellular GTP by 40% by treatment of β-cells with mycophenolic acid also had no effect on the KATP channel-independent pathway when studied under the KCl-diazoxide paradigm (28). These data appear to preclude a major controlling role for adenine and guanine nucleotides in the KATP channel-independent pathway.

In conclusion, in the presence of elevated [Ca\(^{2+}\)]\(_i\), activation of GDH and increased activity of the TCA cycle provide all the signals necessary for activation of the KATP channel-independent pathway. Neither decreased fatty acid oxidation nor esterification into lipids is necessary for BCh to mimic the effect of glucose on the pathway. The nature of the signals emanating from the TCA cycle that augment insulin secretion via the KATP channel-independent pathway are unknown and remain to be determined. With respect to the effect of the signals, our hypothesis is that they accelerate the rate at which docked granules achieve the immediately releasable state (52).

DISCLOSURE

The work described herein was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-28348 to M. J. Macdonald, by Grants DK-54243 and DK-56737, and by a Mentor-Based Postdoctoral Fellowship from the American Diabetes Association to G. W. G. Sharp, and by a Career Development Award from the Juvenile Diabetes Research Foundation International to S. G. Straub.

REFERENCES


