Activation of the K\textsubscript{ATP} Channel-Independent Signaling Pathway
by the non-hydrolysable analog of leucine, BCH.

by

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Running title: BCH activates the K\textsubscript{ATP} channel-independent pathway

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Abstract.

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 KATP channel-independent pathway was studied in the presence of diazoxide and KCl, leucine and its non-metabolizable analog BCH both stimulated insulin secretion to a greater extent than glucose. Glutamine and dimethyl-glutamate had no effect. As the only known action of BCH is stimulation of glutamate dehydrogenase, this is sufficient to develop the full effect of the KATP channel-independent pathway. Glucose, leucine and BCH had no effect on intracellular citrate levels. Leucine and BCH both decreased glutamate levels while 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 KATP 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 KATP channel-independent pathway.
At least two major signaling pathways are involved in glucose-stimulus secretion coupling. These are the KATP channel-dependent and KATP 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 (27, 54). The KATP channel-dependent pathway closes ATP-sensitive K⁺ (KATP) channels (4, 13). This results in depolarization, Ca²⁺ entry via voltage-gated Ca²⁺ channels, raised [Ca²⁺]i and the stimulation of secretion. The KATP channel-independent pathway (6, 18, 48) works in synergy with the KATP channel-dependent pathway because of a requirement for increased [Ca²⁺]i. It should be noted however that simultaneous activation of PKA and PKC circumvents the Ca²⁺ 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 KATP channel-dependent pathway are well-defined (1, 4, 13, 23), the mechanisms involved in the KATP channel-independent pathway are not yet known and are subject to considerable controversy.

Four current hypotheses regarding the KATP channel-independent pathway are as follows. 1. Glucose, as a result of anaplerosis, induces an increase in mitochondrial citrate, cytosolic citrate, and cytosolic malonyl-CoA. The resulting malonyl-CoA-induced inhibition of CPT1 and diversion of fatty acids away from oxidation results 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 production of glutamate in the mitochondria, which is exported to the cytosolic compartment. In the cytosol, glutamate sensitizes the secretory machinery to Ca²⁺ perhaps by an action on the granules (38). 3. Changes in adenine nucleotide concentrations are regulators of the KATP channel-independent pathway (49). 4. Protein acylation is involved (52, 56).

In the present study, we re-examined the idea that only nutrients can activate the KATP 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 non-metabolizable 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 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 sole mechanism by which it stimulates insulin secretion (16, 20, 27, 39, 43, 50). The provision of leucine and glutamine mimicks the pyruvate dehydrogenase and pyruvate carboxylase pathways of glucose metabolism respectively by providing mitochondrial acetyl-CoA and ATP, and mitochondrial intermediates (anaplerosis) respectively (see Figure 1). The combination of these two compounds reproduced the effect of glucose to activate both the KATP channel-dependent
and K<sub>ATP</sub> channel-independent pathways. Most importantly, it was found that the non-nutrient BCH alone (without glutamine) was sufficient to replicate the effect of glucose on the K<sub>ATP</sub> 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 TCA cycle as key activators of the K<sub>ATP</sub> channel-independent pathway.

**Materials and Methods**

**Materials:**

Glucose, leucine, glutamine, BCH, L-carnitine, diazoxide and etomoxir were obtained from Sigma (St. Louis, MO). [1-<sup>14</sup>C]-palmitate and <sup>125</sup>I-insulin were obtained from New England Nuclear Life Science Products Inc., Boston, MA.

**Isolation of pancreatic islets:**

Male Sprague-Dawley rats (250-350g) were used in this study and had access to unlimited food and water. After CO<sub>2</sub> asphyxiation, the pancreas was surgically removed and the islets isolated by collagenase digestion (31).

**Insulin secretion under perifusion conditions:**

Krebs-Ringer bicarbonate-HEPES buffer (KRBH) containing (in mM) 129 NaCl, 5 NaHCO<sub>3</sub>, 4.8 KCl, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 2.5 CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub>, 10 HEPES) at pH 7.4 and 0.1 % bovine serum albumin 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 minute intervals and a flow rate of 1 ml/min for RIA using a charcoal separation method (21). The nutrient and BCH concentrations in the various experiments are specified in the legends and the term basal glucose refers to a concentration of 2.8 mM glucose. When the K<sub>ATP</sub> channel-independent pathway was studied, 250 µM 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 oxidation:**

The method was as previously reported (12, 56). In brief, paired groups of 15 islets were incubated at 37°C for 1 hour in 200 µl of KRBH buffer, to which were added 0.2 µCi [1-<sup>14</sup>C] palmitate, 0.8 mM L-carnitine, BSA (to a final concentration of 1%) and test agents as indicated. At the end of the incubations, islet metabolism was stopped by the addition of 200 µl of 0.5 M...
HCl to the incubation mixture. $^{14}\text{CO}_2$ was collected in 300 µl of 1 M NaOH and measured by liquid scintillation spectrometry.

**Measurement of palmitate incorporation into lipids:**
The method is 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 KRBH containing 0.8 mM L-carnitine, 0.2 µCi [1-$^{14}$C] palmitate and the test compounds (glucose, BCH, leucine, diazoxide and KCl) in Eppendorf tubes in a 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 ice-cold PBS. Immediately after addition of 200 µl of 0.2 M NaCl, the islet pellets were frozen in liquid N$_2$. After thawing, 750 µl of chloroform:methanol (2:1) and 50 µl of 0.1 N KOH were added and the samples vortexed vigorously for 2 min. After centrifugation at 2000 g for 20 min the bottom lipid-soluble layer was washed once with 200 µl of methanol:water:chloroform (48:47:3) and 200 µl of this phase was added to 5 ml BioSafe2 scintillation mixture and incorporation of radiolabeled palmitate into lipids quantified by liquid scintillation counting.

In some experiments, islet samples were incubated for 60 min in the presence of test substances, supplemented with L-carnitine and 0.2 µCi [1-$^{14}$C] palmitate as described above. After 2 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 $\text{H}_2\text{O}$ was 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 nitrogen. 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 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 BioSafe2 scintillation fluid, radioactivity was determined by liquid scintillation counting.

**Metabolite assays:**
Batches of 100 rat islets were incubated for 30 minutes 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 Figure 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 minutes after exposure to glucose. A nadir after 8 minutes was followed by a rising second phase, which reached a plateau after 20 – 30 minutes. 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, while 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.

In order 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 Figure 2B. Glucose and glutamine gave similar data to those in Figure 2A. Interestingly, BCH reproduced the effect of leucine both alone and in combination with glutamine. Glutamine was without effect on secretion, while the combination of glutamine and leucine 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 glutamine, on the K_ATP channel-independent pathway of β-cell signaling (see Figure 3). In this paradigm, the first phase of the glucose response is mimicked by the depolarizing action of KCl while the K_ATP channel-independent pathway provides the second phase. The response to glucose and KCl was slightly biphasic with a peak response after 5 minutes, a slight nadir and a subsequently rising second phase. The responses to leucine plus glutamine, and BCH plus glutamine in the presence of KCl and diazoxide were similar to each other and both stimulated biphasic insulin secretion to a significantly greater extent than glucose.
Again similar results were obtained when dimethyl glutamate was used instead of glutamine (data not shown). Thus activation of the TCA cycle by provision of substrate and activation of GDH mimics the effect of glucose on the K<sub>aTP</sub> channel-independent pathway. The greater response seen with the combination of glutamine or dimethyl glutamate and either leucine or BCH compared with that of glucose is likely due to the activation of GDH by leucine and BCH and the resulting greater influx of α-ketoglutarate in the TCA cycle.

**Figure 3 here**

In a third series of experiments, leucine, BCH, and glutamine alone were tested for their effects on the K<sub>aTP</sub> channel-independent pathway. The results are shown in Figure 4. Remarkably, over the first 20 minutes (minute 10 to 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, while the response to leucine was slightly, but not significantly, less than that of glucose.

While 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 as the effects of glutamine plus KCl or dimethyl glutamate plus KCl were the same as KCl alone.

**Figure 4 here**

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

**Figure 5 here**

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 Figure 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). 20 mM BCH slightly but under these conditions non-significantly increased palmitate oxidation (11%, P>0.05). The importance of these data is that BCH, of all the compounds tested, failed to decrease palmitate oxidation despite its similar effect on insulin secretion and ability to stimulate the KATP 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 and glutamine were examined, palmitate oxidation was reduced 58% by 10 mM glutamine (P<0.01); 64% by 20 mM leucine plus 10 mM glutamine (P<0.001); and 41% by 20 mM BCH and 10 mM glutamine (P<0.05). In the presence of 40 mM KCl and 250 µM diazoxide, palmitate oxidation was reduced 61% by glutamine (P<0.001); 61% by leucine plus glutamine (P<0.001); and 49% by BCH and glutamine (P<0.01). The results are shown in Figure 6.

Figure 6 here

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 Figure 7. 16.7 mM glucose 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 µM diazoxide (again P<0.005 for both glucose and leucine).

Figure 7 here

Additional studies were performed in an attempt to detect small changes that might have been obscured by the method that measures the incorporation of [14C]-palmitate into total lipids. Thus, after labeling the islets in the presence of [14C]-palmitate and the same stimuli as in Figure 7 over a 30 minute 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. Leucine increased the incorporation into phospholipids (P<0.001), monoacylglycerols (P<0.05) and
triglycerides (P<0.001), but not into diacylglycerols. 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 KATP channel-independent pathway involving increased DAG synthesis (57) and activation of DAG binding proteins such as Munc-13 that are involved in and can augment exocytosis (9, 11, 46). The esterification results are shown in Figure 8.

**Figure 8 here**

In seeking for additional insight into the results obtained, intracellular levels of malate, citrate, glutamate and α-ketoglutarate were determined. The results are presented in Table 1. In HIT-T15 cells, the increase in citrate and malonyl-CoA levels during glucose-stimulated insulin release results in inhibition of CPT1, diversion of long chain acyl-CoAs from oxidation in the mitochondria, and presumed increased levels of acyl-CoA in the cytosol (10). We found no change of citrate levels in rat pancreatic islets in response to any of the secretagogues studied (Table 1). BCH and leucine decreased glutamate levels in the absence or presence of KCl and diazoxide, presumably due to the allosteric activation of glutamate dehydrogenase and enhanced conversion of glutamate to α-ketoglutarate (37). In line with this, α-ketoglutarate levels were increased by BCH. Since BCH does not stimulate insulin release by its own metabolism, the decrease in glutamate and increase in α-ketoglutarate levels reflects the increased metabolism of endogenous fuel required to supply the TCA cycle, the KATP channel-independent signaling pathway and subsequent exocytosis. As diazoxide, which maintains the KATP channel in the open state, did not prevent the alterations in metabolite levels, the changes induced by BCH were independent of KATP channel effects. Incubation with 10 mM glutamine, that alone has no effect upon insulin secretion, increased glutamate levels eight-fold consistent with previous results (37).

Finally, to address the question as to whether increased TCA flux alone, or long chain acyl-CoA accumulation alone is sufficient to recapitulate the KATP channel-independent pathway, the effect of the CPT1 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 mM, 10 mM or 20 mM BCH. In these experiments, 10 _M 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 3mM BCH (control 1.92±0.18 % of content released per 60 min vs. 2.24±0.33 %, n=9), 10 mM BCH (3.72±0.61 % of content released per 60 min vs. 3.84±0.56 %, n=5), or 20 mM BCH (4.26±0.38 % of content released per 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
Discussion

The biphasic insulin secretory response to glucose is mimicked by the combination of leucine and glutamine (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:ADP ratio leads to increased $[\text{Ca}^{2+}]_i$, and the first phase of the secretory response. Increased TCA cycle activity produces the signals that synergize with the increased $[\text{Ca}^{2+}]_i$ and induce the second phase. The fact that the combination of the non-metabolizable 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 KATP 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 KATP channels opened by diazoxide, KCl elevates $[\text{Ca}^{2+}]_i$ to provide a “first phase” of insulin secretion and the elevation of $[\text{Ca}^{2+}]_i$ necessary for synergy with signals from the KATP 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 KATP channel-independent pathway than did glucose. Next we determined whether the KATP channel-independent pathway under these conditions was being stimulated by glutamine alone, by leucine and BCH activation of glutamate dehydrogenase 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 non-metabolizable leucine analog BCH not only mimicked but exceeded the effect of glucose. It is clear from the BCH data presented in Figure 4 that in the presence of increased $[\text{Ca}^{2+}]_i$, activation of the TCA cycle by activation of glutamate dehydrogenase is all that is required for the full expression of the KATP channel-independent pathway of β-cell signaling. Enhanced $\text{Ca}^{2+}$ influx not only leads to a rise in cytosolic $\text{Ca}^{2+}$ but also to a rise in intra-mitochondrial $\text{Ca}^{2+}$ thereby activating $\text{Ca}^{2+}$-sensitive enzymes such as isocitrate dehydrogenase and α-ketoglutarate dehydrogenase. While these enzymes can act in the absence of $\text{Ca}^{2+}$, their activity is markedly enhanced by $\text{Ca}^{2+}$ (40,41).

These results emphasize the critical role of the mitochondria in generating the signals necessary for stimulation of insulin secretion via the KATP channel-independent pathway. Both

released per 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 CPT1 and long chain acyl-CoA accumulation is insufficient to reproduce the effects of the KATP channel-independent pathway. In contrast, activation of the TCA cycle is sufficient.
anaplerosis and activation of glutamate dehydrogenase 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 KATP channel-independent pathway. In support of this, succinate (used as the methyl ester) also induces biphasic insulin release and strongly stimulates the KATP 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 KATP 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 KATP 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 CPT1 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, 58). 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). Secondly, 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 KATP channel-independent pathway (22, 42, 53) and which may display different metabolic pathways to those of the native β-cell. With respect to the data in the current paper, we could not show an increase in citrate levels in response to glucose or other combinations of agents that stimulate the KATP channel-independent pathway. Furthermore, BCH that strongly activates this pathway fails to reduce palmitate oxidation or to increase palmitate incorporation into lipids as does glucose. While 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) remains strong and is supported by our data.

A second hypothesis states that glucose induces an increase in the production of glutamate in the mitochondria, which is exported to the cytosolic compartment and sensitizes the secretory machinery to Ca²⁺ (38). This hypothesis is also controversial (5, 37, 55). 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\textsuperscript{2+}-stimulated insulin secretion. Note again, however that the INS-1 cell used does not manifest the K\textsubscript{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 while the secretory response to KCl was unaffected. Similar results were obtained in perifused rat pancreatic islets following adenovirus transfection of GAD (47). The evidence against this hypothesis is that we 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 (55). 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 K\textsubscript{ATP} channel-independent pathway (49). However, the activity of the K\textsubscript{ATP} channel-independent pathway was unaltered under conditions in which the ATP/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/ADP ratio relative to control islets. This defect was presumed responsible for the impaired secretion because of the failure of glucose to decrease K\textsubscript{ATP} channel activity and depolarize the β-cell. When the effect of glucose via the K\textsubscript{ATP} 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 K\textsubscript{ATP} 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 K\textsubscript{ATP} channel independent pathway when studied under the KCl/diazoxide paradigm (30). These data appear to preclude a major controlling role for adenine and guanine nucleotides in the K\textsubscript{ATP} channel-independent pathway.

In conclusion, in the presence of elevated [Ca\textsuperscript{2+}]\textsubscript{i}, activation of GDH and increased activity of the TCA cycle provide all the signals necessary for activation of the K\textsubscript{ATP} channel-independent pathway. Neither decreased fatty acid oxidation nor esterification into lipids is necessary for BCH to mimick the effect of glucose on the pathway. The nature of the signals emanating from the TCA cycle that augment insulin secretion via the K\textsubscript{ATP} 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 (53).

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Figure Legends

Figure 1.
Scheme to illustrate the pathways by which leucine and glutamine feed into the pyruvate dehydrogenase and pyruvate carboxylase pathways of glucose signaling. These two pathways provide acetyl-CoA and ATP, and TCA cycle intermediates respectively and can be associated with the first and second phases of glucose stimulated insulin secretion.

Figure 2.
A. The effects of 16.7 mM glucose, 20 mM leucine, 10 mM glutamine, and the combination of 20 mM leucine and 10 mM glutamine on insulin secretion from rat islets, (n=6). When the 16.7 mM glucose-stimulated insulin release from the nadir to the end of the experiment was integrated it averaged 103±6 pg/islet/min. 20 mM leucine–stimulated insulin release averaged 64±5 pg/islet/min, Δ = -39±7 pg/islet/min, P<0.001. B. The effects of 16.7 mM glucose, 20 mM BCH, 10 mM glutamine, and the combination of 20 mM BCH and 10 mM glutamine on insulin secretion from rat islets, (n=6). 16.7 mM glucose-stimulated insulin release integrated from the nadir to the end of the experiment averaged 124±5 pg/islet/min. 20 mM BCH–stimulated insulin release averaged 65±6 pg/islet/min, Δ = -59±9 pg/min/islet, P<0.001, n=6.

Figure 3.
The effects of 16.7 mM glucose, and the combinations of 20 mM leucine and 10 mM glutamine, and 20 mM BCH and 10 mM glutamine, on insulin secretion from rat islets under the KATP channel independent paradigm in the presence of 250 µM diazoxide and 40 mM KCl (n=6). Over the first phase period, glucose-stimulated insulin secretion averaged 80±4 pg/islet/min and leucine + glutamine-stimulated release 125±6 pg/islet/min, Δ = 45±9 pg/islet/min, P<0.001, n=6. BCH + glutamine-stimulated release averaged 128±5 pg/islet/min, Δ (vs. glucose) = 48±10 pg/islet/min, P<0.001, n=6. Over the second phase, glucose-stimulated insulin secretion averaged 126±3 pg/islet/min and leucine + glutamine-stimulated release 183±12 pg/islet/min, Δ = 57±6 pg/islet/min, P<0.001, n=6. BCH+glutamine-stimulated release averaged 183±11 pg/islet/min, Δ (vs. glucose) = 57±6 pg/islet/min, P<0.01, n=6.

Figure 4.
The effects of 16.7 mM glucose, 20 mM leucine, 20 mM BCH, and 20 mM glutamine on insulin secretion from rat islets under the KATP channel-independent paradigm in the presence of 250 µM diazoxide and 40 mM KCl (n=6). Over the first 20 minutes (minute 10 to 30), leucine and BCH alone stimulated insulin secretion via the KATP channel-independent pathway to a
significantly greater extent than glucose (Glucose 124±7 pg/islet/min; leucine 180±9 pg/islet/min, Δ = 56±7, P<0.001; BCH 196±9 pg/islet/min, Δ (vs. glucose) = 72±9 pg/islet/min, P<0.001, n=6).

**Figure 5.**
The effects of 10 mM succinic acid methylester and 16.7 mM glucose on insulin secretion from rat islets under normal conditions (panel A) and under the KATP channel-independent paradigm (panel B) in the presence of 250 μM diazoxide and 40 mM KCl (n=7).

**Figure 6.**
The effects of 16.7 mM glucose, 20 mM leucine, 10 mM BCH, 10 mM glutamine, 20 mM leucine plus 10 mM glutamine, and 20 mM BCH plus 10 mM glutamine on [1-14C] palmitate oxidation in the absence (left hand panel) and presence (right hand panel) of 250 μM diazoxide and 40 mM KCl (n=5). * indicates a significant decrease in palmitate oxidation. Δ indicates a significant increase in oxidation. The levels of significance are stated in the text.

**Figure 7.**
The effects of 16.7 mM glucose, 20 mM BCH and 20 mM leucine on [1-14C] palmitate incorporation into lipids in the absence and presence of 250 μM diazoxide and 40 mM KCl (n=8). * indicates a significant increase in incorporation (P<0.005).

**Figure 8.**
The effects of 16.7 mM glucose, 20 mM leucine and 20 mM BCH on [1-14C] palmitate incorporation into phospholipids, and into mono-, di-, and triacylglycerides in the presence of 250 μM diazoxide and 40 mM KCl (n=3). The asterisks indicate significant increases in incorporation (* = P<0.05; ** = P<0.001).
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. Control is KRBH with 2.8 mM glucose. Results are expressed as means ± SE with the number of replicate incubations in parentheses.

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Level of Metabolite in Islets (nmol metabolite / mg islet protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Malate</td>
</tr>
<tr>
<td>No addition</td>
<td>2.1 ± 0.2 (6)</td>
</tr>
<tr>
<td>BCH</td>
<td>2.2 ± 1.0 (6)</td>
</tr>
<tr>
<td>Leucine</td>
<td>2.3 ± 0.2 (4)</td>
</tr>
<tr>
<td>KCl</td>
<td>2.4 ± 0.6 (4)</td>
</tr>
<tr>
<td>Diazoxide</td>
<td>2.0 ± 0.7 (4)</td>
</tr>
<tr>
<td>BCH, KCl, Diazoxide</td>
<td>2.1 ± 0.2 (4)</td>
</tr>
<tr>
<td>Leucine, KCl, Diazoxide</td>
<td>2.7 ± 0.4 (4)</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.7 ± 0.6 (6)</td>
</tr>
<tr>
<td>Glutamine</td>
<td>4.5 ± 0.8 (4)a</td>
</tr>
</tbody>
</table>

a P<0.05, b P<0.01, c P<0.001 vs no addition
Glucose Metabolism

Leucine → PDH → Pyruvate → PC

Acetyl-CoA → ATP/ADP → KATP channel → [Ca^{2+}]_i

Metabolic coupling factor(s) → Glutamate → Glutamine

INSULIN SECRETION
A

Insulin Release (pg/islet/min)

0 50 100 150 200

Minutes

B

Insulin Release (pg/islet/min)

0 50 100 150 200

Minutes

- ■ 16.7 mM Glucose
- ◇ 20 mM Leucine
- ▲ 10 mM Glutamine
- ○ 20 mM Leucine + 10 mM Glutamine
- 16.7 mM Glucose
- ▲ 10 mM Glutamine
- △ 20 mM BCH + 10 mM Glutamine
- △ 20 mM BCH
- ▲ 10 mM Glutamine
- ○ 20 mM Leucine + 10 mM Glutamine
Insulin Release (pg/islet/min)

- KCl + diazoxide
- 16.7 mM Glucose
- 20 mM BCH + 10 mM Gln
- 20 mM Leucine + 10 mM Gln

Minutes
Insulin Release (pg/islet/min)

KCl + diazoxide

- ■ 16.7 mM Glucose
- ◊ 20 mM Leucine
- ▲ 20 mM Glutamine
- □ 20 mM BCH

Minutes

0 10 20 30 40 50 60 70

0 50 100 150 200 250 300
Insulin Release (pg/islet/min)

Minutes

25 100 200 300 400

0 2 0 3 0 5 0

KCl + diazoxide

10 mM SAME

16.7 mM Glucose

10 mM SAME

16.7 mM Glucose

Minutes
[1-14C] palmitate -> CO₂ (pmol/islet * 60 min)

- 2.8 mM glucose
- 16.7 mM glucose
- 20 mM BCH
- 20 mM Leucine
- 10 mM Gln
- BCH + Gln
- Leucine + Gln

KCl + diazoxide

* Denotes statistical significance compared to control.
[1-14C] palmitate -> lipid (pmol/islet * min)

KCl + diazoxide

2.8 mM glucose
16.7 mM glucose
20 mM BCH
20 mM Leucine
Phospholipids
MAG's
DAG's
Triglycerides

[1-14C] palmitate -> lipids
(pmol/100 islets * 30 min)

KCl + diazoxide

* 2.8 mM glucose
** 16.7 mM glucose
Leucine
BCH

Phospholipids  MAG's  DAG's  Triglycerides