Anaplerotic input is sufficient to induce time-dependent potentiation of insulin release in rat pancreatic islets

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Gunawardana, Subhadra C., Yi-Jia Liu, Michael J. MacDonald, Susanne G. Straub, and Geoffrey W. G. Sharp. Anaplerotic input is sufficient to induce time-dependent potentiation of insulin release in rat pancreatic islets. Am J Physiol Endocrinol Metab 287: E828–E833, 2004; doi:10.1152/ajpendo.00381.2003.—Nutrients that induce biphasic insulin release, such as glucose and leucine, provide acetyl-CoA and anaplerotic input in the β-cell. The first phase of release requires increased ATP production leading to increased intracellular Ca2+ concentration ([Ca2+]i). The second phase requires increased [Ca2+]i and anaplerosis. There is strong evidence to indicate that the second phase is due to augmentation of Ca2+-stimulated release via the KATP channel-independent pathway. To test whether the phenomenon of time-dependent potentiation (TDP) has similar properties to the ATP-sensitive K+ channel-independent pathway, we monitored the ability of different agents that provide acetyl-CoA and anaplerotic input or both of these inputs to induce TDP. The results show that anaplerotic input is sufficient to induce TDP. Interestingly, among the agents tested, the nonsecretagogue glutamine, the nonhydrolyzable analog of leucine aminoisobutyric acid (Glutamine), and succinic acid methyl ester all induced TDP, and all significantly increased α-ketoglutarate levels in the islets. In conclusion, anaplerosis that enhances the supply and utilization of α-keto-glutarate in the tricarboxylic acid cycle appears to play an essential role in the generation of TDP.

insulin secretion; anaplerosis; α-ketoglutarate

TIME-DEPENDENT POTENTIATION (TDP), often termed priming, can be defined as the enhancement of a secretory response in the β-cell due to a memory induced by a previous exposure to certain secretagogues. TDP has been observed in in vitro preparations of islets and pancreases from different species, including rat (17–19, 21, 55–57), rabbit (15), and spiny mouse (37), as well as in in vivo studies in humans (4–7, 39). TDP occurs simultaneously with time-dependent inhibition (TDI) but lasts longer than TDI (5, 11, 38, 39). TDP and TDI appear to be acute effects of relatively short exposures to high glucose, not related to the third phase of insulin secretion, i.e., desensitization caused by prolonged exposure to glucose (23). Increased sensitivity to glucose is observed after 3 h of exposure to glucose, whereas >6 h of exposure leads to desensitization (43). The physiological importance of TDP is clear. In non-insulin-dependent diabetes mellitus, where the direct insulin response to glucose is impaired, the potentiating function of glucose remains intact. In some diabetic subjects, the defective insulin response to glucose can be restored to normal by inducing TDP (7, 37, 39). In some animal models of diabetes, both the acute phase of insulin release and TDP are impaired (20, 22, 36), and the correction of the defect in the TDP pathway may correct the defect in direct insulin release.

Glucose is the best studied physiological priming agent, whereas other nutrients, such as glyceraldehyde, methyl pyruvate, leucine, and α-ketoisocaproate (α-KIC) also induce priming (18, 19, 55–57). The underlying mechanism is not known. The information available on this subject includes that 1) TDP results in a general enhancement of the secretory response to all secretagogues (6, 21); 2) glucose-induced TDP requires the metabolism of glucose (19); 3) TDP is not dependent on insulin biosynthesis, elevation of cAMP, or ATP-sensitive K+ (KATP) channel function (19, 50, 51); and 4) TDP may involve cellular phosphoinositide metabolism (56). The requirement of extracellular Ca2+ for TDP has been a subject of some controversy, the majority of studies indicating that Ca2+ is essential for glucose-induced TDP, with only a few reports to the contrary. Recent work has demonstrated that glucose-induced TDP is critically dependent on intracellular pH (24). Although Ca2+ is permissive for glucose-induced priming under physiological conditions, an intracellular H+ concentration ([H+]i) above 63 mmol/l (pH 7.2) can override the requirement for Ca2+ and enable glucose to induce priming under stringent Ca2+-free conditions. Furthermore, if the [H+]i is decreased below 20 mmol/l (pH 7.7), the priming ability of glucose is compromised despite the presence of Ca2+ (24).

On the basis of the facts that 1) glucose metabolism is a prerequisite for glucose-induced priming and 2) all nutrients known to induce TDP are metabolized through the mitochondria, it seems likely that the priming signals are generated by mitochondrial metabolism. Augmentation of insulin release, a related function that shares common steps with TDP in the rat, is also produced by nutrients that accelerate mitochondrial metabolism. There is strong evidence that anaplerosis plays an important role in the stimulation of insulin release (2, 8, 31, 42) and that it may be the sole requirement for the augmentation of Ca2+-stimulated insulin release by the KATP channel-independent pathway (31).

To establish whether mitochondrial metabolism plays an equally important role in TDP, we examined the magnitude of priming induced by various nutrients and aminobicyclo[2.2.1]-heptane-2-carboxylic acid (BCH) that are known to provide acetyl-CoA and/or anaplerotic input. Our results demonstrate that anaplerotic input alone is sufficient to induce TDP. These results further suggest that the priming signal may be generated...
in a specific area of the tricarboxylic acid (TCA) cycle, namely the reactions that involve the generation and utilization of α-ketoglutarate (α-KG). The enzymes that catalyze these reactions are glutamate dehydrogenase (GDH) and isocitrate dehydrogenase (ICDH), which generate α-KG, and α-ketoglutarate dehydrogenase (α-KGDH), which converts α-KG to succinyl-CoA. These enzymes in several tissues are activated by low pH and/or Ca²⁺ (9, 26, 32, 34, 35, 40, 41), as is TDP in the β-cell (24).

RESEARCH DESIGN AND METHODS

Islets. Freshly isolated islets from 2- to 3-mo-old male Wistar rats were used. Islets were isolated using the method of Lacy and Kostianovsky (29) with minor modifications. The animals, obtained from Harlan Laboratories (Indianapolis, IN), were cared for according to the guidelines of the Institutional Animal Care and Use Committee at Cornell University and fed ad libitum with standard rat chow.

Media. Krebs-Ringer-bicarbonate solution of pH 7.4, buffered with 10 mmol/l HEPES (KRBH), was used for islet isolation and all incubations. The standard composition of the KRBH solution was (in mmol/l) 128.8 NaCl, 4.8 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 2.5 CaCl₂, 5 NaHCO₃, and 10 HEPES. In the Ca²⁺-free KRBH medium used during the priming period of certain indicated experiments, Ca²⁺ was omitted from the medium, and 5 mmol/l EGTA were added to ensure stringent Ca²⁺-free conditions. Although it is possible that some Ca²⁺ remains in the cellular organelles even with EGTA treatment, it is not large enough to influence the priming ability of nutrients. We have also shown that glucose and leucine can induce strong TDP under conditions where both extracellular and intracellular Ca²⁺ are depleted using EGTA and BAPTA-AM, a chelator of intracellular Ca²⁺ (data not shown). The magnitude of TDP was similar in both the presence and absence of BAPTA-AM. The basal glucose concentration used was 2.8 mmol/l, and the priming glucose concentration was 11.1 mmol/l in Ca²⁺-free conditions and 16.7 mmol/l in the presence of Ca²⁺. These are the optimal concentrations of glucose for the stimulation of insulin secretion in the absence and presence of Ca²⁺, respectively (27), and for priming (data not shown). During all incubations, 0.1% bovine serum albumin (BSA) was present in the medium. Stimulation of islets in the test period, as distinct from the priming period, was always performed with 16.7 mmol/l glucose in the presence of Ca²⁺.

Static incubations. Freshly isolated islets were preincubated for 30 min in KRBH with 2.8 mmol/l glucose. The islets were then subjected to a 45-min priming period in the presence of different agents, such as glucose, leucine, glutamine, α-KIC, BCH, succinic acid methyl ester (SAME), palmitate, and myristate, as indicated in each experiment. Groups of control islets were maintained in KRBH containing 2.8 mmol/l glucose throughout the priming period. The priming was carried out in either the presence or absence of Ca²⁺ as indicated. Except for the priming period in certain indicated experiments, all incubations were performed in the presence of 2.5 mmol/l Ca²⁺. After the priming period, the islets were washed and “rested” in KRBH containing 2.8 mmol/l glucose for 20 min, followed by the test stimulation with 16.7 mmol/l glucose for 20 min. At the end of the test period, samples were collected to measure the rates of insulin secretion and the islet insulin contents. All incubations were conducted at 37°C. In each static incubation experiment, three to five replicate batches of five size-matched islets were used for each priming condition; n represents the number of times each experiment was repeated with separate batches of islets from individual rats. The aforementioned time periods for priming and rest were selected through experience from preliminary work and using the following information from the literature. TDP is known to occur following exposures to glucose ranging from 5 to 60 min in duration, with rest periods also ranging from 5 to 60 min (17, 39). However, the magnitude of TDP is smaller with short exposures <10 min, due to the concurrent TDI, which is overcome with longer exposures to glucose (5, 11, 39). A 30- to 45-min exposure to glucose appears to be optimal for induction of TDP, and the magnitude decreases slightly with longer exposures, probably due to the general run-down of the secretory ability of isolated islets with time in vitro (17). Although TDP was observed after a 60-min rest period before the second pulse of glucose, it was lost after a 90-min rest period (17, 39).

Insulin measurement. Radioimmunoassay was used to measure the amount of insulin released during the final stimulation and the total content of insulin in the islets. Values are expressed as fractional release, i.e., as a percentage of the total insulin content of the islets released per 20 min. The total islet insulin contents are included in the figure legends.

Measurement of α-KG. Batches of 100 islets were incubated in KRBH containing 2.8 mM glucose and 0.1% BSA at 37°C for 30 min. They were then exposed for 45 min to KRBH containing 2.8 mM glucose alone or with 20 mM BCh, 20 mM BCH plus 10 mM glutamine, or 20 mM SAME. At the end of this priming period, α-KG was measured by alkali-enhanced fluorescence of NAD(P)(H) as described previously (31, 33).

Statistical analysis. Values are expressed as means ± SE. The data were assessed by ANOVA and Student’s t-test, paired and unpaired, as appropriate.

Materials. Glucose, leucine, glutamine, BCH, and dimethyl amiloride (DMA) were obtained from Sigma (St. Louis, MO). 125I-labeled insulin was obtained from New England Nuclear Life Science Products (Boston, MA).

RESULTS

To investigate the role of mitochondrial metabolism in TDP, we examined the magnitude of TDP induced by a number of mitochondrial fuels that provide anaplerotic input, acetyl-CoA production, or both.

Compounds such as leucine and α-KIC that produce both anaplerotic input and acetyl-CoA induced strong TDP, similar to glucose. The induction of TDP by leucine compared with that of glucose is shown in Fig. 1 in the presence (Fig. 1A) and absence (Fig. 1B) of extracellular Ca²⁺. Similar TDP was induced by α-KIC (data not shown). TDP induced by several anaplerotic compounds that activate and enter the TCA cycle at different sites was also monitored. Glutamine, a noninsulinotropic amino acid that enters the TCA cycle after metabolism to glutamate and α-KG, induced a moderate degree of TDP in the presence and absence of extracellular Ca²⁺ (Fig. 1, A and B). The combination of leucine and glutamine induced a greater degree of priming than either agent alone in both situations, thus exhibiting a similar behavior to the effect of these agents on the stimulation of insulin release (31, 44, 45, 47). Increasing the concentration of leucine from 20 to 30 mmol/l did not enhance the magnitude of priming by leucine, indicating that the augmenting effect of glutamine was not through compensation for a suboptimal concentration of leucine (data not shown). Although leucine can stimulate insulin release only above a threshold concentration between 3 and 5 mmol/l (47), it can enhance glucose-induced insulin release even at the physiological concentration of 0.25 mmol/l (46). However, most studies on insulin secretion have used these compounds (leucine, KIC, BCH, and succinate) at concentrations of 10–20 mmol/l. We selected 20 mmol/l, because this concentration induces the maximal effect on both secretion and TDP (slightly greater than 10 mmol/l).
Fig. 1. Priming induced by agents that provide acetyl-CoA and/or anaplerotic input in the presence (A) and absence (B) of extracellular Ca\(^{2+}\). Insulin release in response to 16.7 mmol/l glucose is shown in paired islets previously exposed to 16.7 mmol/l glucose, leucine, glutamine, or leucine + glutamine. Incubations in the priming period were conducted in Ca\(^{2+}\)-containing Krebs-Ringer bicarbonate-HEPES (KRHB) for experiments in A and in Ca\(^{2+}\)-free KRHB containing 5 mmol/l EGTA for experiments in B. Gln, 20 mmol/l glucose; Leu, 10 mmol/l leucine; Gln, 10 mmol/l glutamine. *P < 0.001 and #P < 0.05 compared with each unprimed control (2.8G); n = 8 for A and n = 6 for B. For results in these figures, the corresponding total insulin contents for each condition (from left to right), expressed as mean ng/islet ± SE, are as follows: A: 37.3 ± 3.7, 34.7 ± 3.3, 38.0 ± 3.3, 30.8 ± 2.9, and 31.4 ± 2.3; B: 44.9 ± 5.3, 63.1 ± 10.2, 51.3 ± 5.1, 43.6 ± 7.6, and 49.4 ± 8.2.

SAME induced strong TDP in both the presence and absence of Ca\(^{2+}\) (Fig. 2, A and B). BCH, the nonmetabolizable analog of leucine, induced priming to the same as extent or greater than glucose in both the presence and absence of Ca\(^{2+}\) (Fig. 3, A and B). Although not metabolized as are the other TDP-inducing agents, BCH is an allosteric activator of GDH (25, 31, 44). The magnitude of BCH-induced TDP shows that the activation of GDH alone is adequate to generate a strong priming signal, thus providing compelling evidence for the importance of α-KG generation in priming.

Other TCA cycle intermediates, such as fumarate and malate (used as methyl esters), did not induce TDP (data not shown). Acetyl-CoA-producing fatty acids, such as palmitate and myristate, which are known to augment insulin release acutely (28, 42, 52, 53), neither induced TDP nor enhanced the priming induced by glucose (data not shown).

While pH sensitivity is shared by the priming induced by glucose (data not shown). Thus it appears that pH sensitivity is shared by the TDP mechanisms of several different nutrients.

All this evidence points toward the requirement of anaplerotic input in the generation of TDP. All the anaplerotic compounds that induced priming shared the ability to supply α-KG to the TCA cycle. In view of this, α-KG was measured in islets under control conditions (45 min with 2.8 mM glucose) or 45 min with three anaplerotic agents. Under control conditions, there was 0.18 ± 0.11 nmol α-KG/mg islet protein
The amount of the amino acid leucine and its metabolite hyde and pyruvate (used experimentally as methyl pyruvate), that do stimulate insulin release could induce TDP. Such induce TDP, it has generally been believed that only agents that do not provide anaplerotic input to the TCA cycle (8, 9). Thus all of these compounds provide anaplerotic input to the TCA cycle and supply α-KG to be metabolized in the α-KGDH reaction. The source of α-KG differs according to the agent, glucose and glyceraldehyde providing α-KG via the ICDH reaction; glutamine, BCH, and succinate via the GDH reaction; and leucine and KIC via both IC heads and CDH and GluDH. All of these compounds induce significant priming. Importantly, the anaplerotic agents BCH and SAME increased α-KG levels in islets more than fourfold. The combination of BCH and glutamine increased α-KG levels in islets 5.4-fold.

TCA cycle intermediates distal to succinate, such as malate and fumarate, did not induce priming. Neither did fatty acids that potentiate insulin release and provide acetyl-CoA but do not provide anaplerotic input. It is noteworthy that none of these compounds activate GDH or α-KGDH. In fact, palmitate is known to inhibit GDH, presumably through active-site acylation (1, 3, 10, 12, 14, 16), an effect that might contribute to its inability to induce TDP. Similarly, malate has been reported to have an inhibitory effect on GDH (3, 12, 14, 16).

The common feature of the priming agents appears to be the ability to provide α-KG in the TCA cycle. However, the possibility that acetyl-CoA plays a role in priming cannot be ruled out. Glucose and leucine directly supply acetyl-CoA, whereas glutamine, BCH, and SAME, through increased TCA cycle activity, may give rise to acetyl-CoA from malate exiting the mitochondria. However, the fact that exogenous fumarate, malate, or fatty acids do not induce priming suggests that acetyl-CoA plays only a supporting role, a role that is essential to the continued activity of the TCA cycle. Nevertheless, in the only study to report on acetyl-CoA levels in islets (30), exposure of rat islets to 25 mM glucose or to 25 mM glucose plus 10 mM glutamate, 10 mM lactate, and 1 mM pyruvate for 30 min resulted in decreased acetyl-CoA levels, suggesting that the levels are being drawn down by the increased TCA cycle activity.

The magnitude of priming induced by BCH, believed to act solely by activating GDH, emphasizes the importance of α-KG in priming. Because BCH induces strong priming, the activa-

DISCUSSION

Although all compounds that stimulate insulin release do not induce TDP, it has generally been believed that only agents that do stimulate insulin release could induce TDP. Such agents include glucose and its metabolites such as glyceraldehyde and pyruvate (used experimentally as methyl pyruvate), the amino acid leucine and its metabolite α-KIC, and the combination of leucine and glutamine. An interesting finding in this study is that priming can be induced by glutamine and by BCH, the latter to a particularly great extent. Glutamine, an amino acid that provides anaplerotic input through conversion to glutamate and α-KG, does not stimulate insulin release by itself (31, 44, 45). The leucine analog BCH is not metabolized in the β-cell and acts only by activating GDH (44). Hence, the ability to stimulate and augment direct insulin release is not essential for priming. SAME is a strong priming agent that does not directly supply acetyl-CoA. Although SAME enters the TCA cycle beyond α-KG, it activates GDH after conversion to succinyl-CoA (13), thus simultaneously providing α-KG to the cycle. Hence, it appears that the provision of α-KG to the TCA cycle is one common mechanism of action shared by all agents known to induce priming.

Each of the priming agents tested enters the TCA cycle as follows. Glucose is converted to pyruvate through glycolytic reactions, and pyruvate enters the TCA cycle as oxaloacetate and acetyl-CoA. Activation of the TCA cycle at this point leads to the production of α-KG through the ICDH reaction. Leucine and α-KIC enter the TCA cycle after conversion to acetyl-CoA, at the citrate synthase step. In addition, they allosterically activate GDH, thereby enhancing the metabolism of endogenous glutamate into α-KG. Glutamine is also metabolized into α-KG via the enzymes glutaminase and GDH. BCH, although nonmetabolizable, provides anaplerotic input by allosteric activation of GDH and increased conversion of endogenous glutamate to α-KG. Succinate, derived from SAME by esterase activity, is acted upon by succinate thiokinase to form succinyl-CoA (13), and succinyl-CoA is also an activator of GDH. Thus all of these compounds provide anaplerotic input to the TCA cycle and supply α-KG to be metabolized in the α-KGDH reaction. The source of α-KG differs according to the agent, glucose and glyceraldehyde providing α-KG via the ICDH reaction; glutamine, BCH, and succinate via the GDH reaction; and leucine and KIC via both ICDH and GluDH. All of these compounds induce significant priming. Importantly, the anaplerotic agents BCH and SAME increased α-KG levels in islets more than fourfold. The combination of BCH and glutamine increased α-KG levels in islets 5.4-fold.

TCA cycle intermediates distal to succinate, such as malate and fumarate, did not induce priming. Neither did fatty acids that potentiate insulin release and provide acetyl-CoA but do not provide anaplerotic input. It is noteworthy that none of these compounds activate GDH or α-KGDH. In fact, palmitate is known to inhibit GDH, presumably through active-site acylation (1, 3, 10, 12, 14, 16), an effect that might contribute to its inability to induce TDP. Similarly, malate has been reported to have an inhibitory effect on GDH (3, 12, 14, 16).

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The magnitude of priming induced by BCH, believed to act solely by activating GDH, emphasizes the importance of α-KG in priming. Because BCH induces strong priming, the activa-
tion of GDH leads to the generation of the priming signal. However, GDH itself cannot be the main physiological reaction that generates a priming signal. This is because glucose, the major physiological priming agent, does not activate GDH. Glucose inhibits GDH through the formation and increase in the concentration of GTP, an allosteric inhibitor of GDH (47, 49). It is likely that it is the supply and utilization of α-KG that is important in generating TDP, whereas the mechanism by which α-KG is produced is not critical. Although BCh may supply α-KG by activating GDH, glucose supplies ample amounts of α-KG through the ICDH reaction, so that its inhibitory effect on GDH would not inhibit the overall α-KG production. Compounds such as glutamine, BCh, and SAME increase the supply of α-KG to the TCA cycle, thereby increasing the cycle activity and utilization of endogenous acetyl CoA.

Intracellular pH plays an important role in TDP, as all the nutrients tested exhibited a pH sensitivity (24). TDP induced by glutamine, normally moderate in magnitude, was markedly enhanced by intracellular acidification with DMA. The magnitude of TDP induced by strong priming agents such as glucose and leucine is further enhanced by DMA, and in situations where glucose normally does not induce TDP (such as in Ca2+-free HCO3−-buffered medium), acidification unmasks the effect (24). Intracellular alkalinization produced by increasing the medium pH inhibits TDP. Thus intracellular pH influences the potentiating ability of different agents that supply α-KG via GDH (e.g., glutamine and leucine) and in addition via ICDH (e.g., glucose). It is well known that α-KGDH is activated by low pH (32, 34, 35, 40), whereas ICDH and GDH are also pH sensitive, the optimum pH varying with the tissue (26, 40, 41). Furthermore, α-KGDH and ICDH are also known to be Ca2+-activated enzymes (9, 34, 35, 40). Hence, it appears that ICDH and/or GDH, which supply α-KG, or the subsequent α-KGDH reaction that utilizes α-KG from both sources may be the critical pH-sensitive steps involved in TDP. Therefore, an enhancement of the activity of these enzymes (produced by Ca2+ and/or H+ ions) may generate the TDP signal via supplying a surplus of α-KG to the TCA cycle, thus priming the TCA cycle for rapid generation of signals at the onset of a subsequent stimulation. As shown by Sener et al. (48), islet α-KGDH is markedly activated by Ca2+ and can retain a memory of previous exposure to glucose. Even though islet α-KGDH is Ca2+ activated, Ca2+ is not essential for its activity so long as adequate concentrations of substrate and cofactors are present. As shown by studies in different tissues (32, 34, 35), α-KGDH is activated by low pH regardless of the presence or absence of Ca2+. Even in the absence of Ca2+ and at a limiting substrate concentration, porcine heart α-KGDH shows considerable activity when the pH is below 7.0, suggesting that an adequate [H+] can override the Ca2+ requirement. The α-KGDH reaction, being a critical common step in the metabolism of all three nutrients tested whose priming ability is pH dependent (glucose, leucine, and glutamine), may well be the major pH-sensitive step in priming.

In summary, the results demonstrate that anaplerotic input alone is sufficient for the induction of TDP. The priming ability of various agents tested here indicates the importance of the reactions that generate and utilize α-KG in the induction of TDP, a concept further supported by the pH/Ca2+ sensitivity of these reactions and of TDP.

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