α-Ketoisocaproate Is Not a True Substrate for ATP Production by Pancreatic β-Cell Mitochondria

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The ability of α-ketoisocaproate (KIC) to induce ATP production in isolated mitochondria from pancreatic β-cells was examined with a bioluminometric method. There was no ATP production from KIC when tested alone or in combination with malate (1 mmol/l), nor did DL-β-hydroxybutyrate induce mitochondrial ATP production, whereas palmitoyl-carnitine and pyruvate were efficient stimulators of mitochondrial ATP production in the presence of an equimolar concentration of malate. However, KIC stimulated the mitochondrial ATP production when tested in combination with glutamate (10 mmol/l). The concentration necessary to obtain half-maximal stimulation was ~50 µmol/l KIC, and maximal activity, comparable to that obtained with fatty acids, was reached at 1 mmol/l KIC. Higher KIC concentrations inhibited the mitochondrial ATP production, whereas a plateau was attained at 1 mmol/l KIC in the presence of glutamine. Ca^{2+} stimulated the maximal mitochondrial ATP production induced by KIC. Maximal stimulation was obtained with 300 nmol/l Ca^{2+} in the presence of 0.3 mmol/l KIC. Ca^{2+} reduced the concentration of KIC necessary for half-maximal stimulation to <30 µmol/l. Leucine stimulated the mitochondrial ATP production in the presence of glutamate to the same extent as KIC. Half-maximal stimulation was observed with 2 mmol/l leucine. There were no additive effects on mitochondrial ATP production when KIC and leucine were tested in combination. The results demonstrate that KIC by itself is not a mitochondrial substrate for ATP production. KIC must transaminate with glutamate or glutamine to yield α-ketoglutarate and leucine. Since leucine allosterically activates glutamate dehydrogenase, which also produces α-ketoglutarate, the insulinogenic effect of KIC may in part be due to the intramitochondrial generation of α-ketoglutarate. Since KIC-induced ATP production reaches a plateau already at micromolar concentrations (i.e., far below the concentrations at which KIC induces insulin release), it is proposed here that the catabolism of KIC may induce additional signals related to insulin release. *Diabetes* 47:339–344, 1998

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Received for publication 8 April 1997 and accepted in revised form 25 November 1997.

DAPP, diadenosine pentaphosphate; KIC, α-ketoisocaproate; D-OHB, D-β-hydroxybutyrate; DL-OHB, DL-β-hydroxybutyrate.

α-Ketoisocaproate (KIC), the deamination product of leucine, is one of the few physiological substrates apart from glucose that initiates a sustained insulin release from the pancreatic β-cell (1,2). Glucose and KIC are decarboxylated in a concentration-dependent manner, increase pyridine nucleotide fluorescence, and hyperpolarize the mitochondrial inner membrane (3–5). These similarities led to the proposal that KIC and glucose initiate insulin release by a common metabolic signal (3). Since KIC is metabolized exclusively in the mitochondria, mitochondrially generated ATP is believed to be the common metabolic link between nutrient stimulation and insulin release from the pancreatic β-cell (4,5). The mitochondrially derived ATP is supposed to close the ATP-dependent K^+ channel in the β-cell plasma membrane (6). The reduced K^+ outflow subsequently depolarizes the membrane potential and initiates Ca^{2+} influx that triggers insulin release (7). Consistent with that model, membrane depolarizations and Ca^{2+} inflow induced by glucose or KIC are very similar (5).

The present study aims at characterizing the KIC-induced ATP production from isolated β-cell mitochondria to scrutinize the hypothesis outlined above. However, the direct study of mitochondrial ATP production from mitochondria isolated from pancreatic β-cells is generally hampered by the restricted tissue availability. ATP production was determined with the recently introduced bioluminometric technique (8) that allows the measurement of mitochondrial ATP production from isolated β-cell mitochondria corresponding to about one islet of Langerhans (i.e., 1 µg of tissue per sample).

RESEARCH DESIGN AND METHODS

Microbial collagenase P (EC 3.4.24.3), firefly luciferase (EC 1.13.12.7), ADP (potassium salt), ATP (potassium salt), DAPP (trilithium salt), and electrophoretically homogeneous, lyophilized bovine serum albumin were purchased from Boehringer (Mannheim, Germany), whereas bovine serum albumin (fraction V) was from Miles Laboratories (Stoke Poges, U.K.). D-Luciferin was purchased from Biothema AB (Dalarö, Sweden). Palmitoyl-carnitine chloride, L-carnitine hydrochloride, L-leucine (free acid), KIC (α-ketoisocaproic acid) (sodium salt), DL-β-hydroxybutyrate (DL-OHB) (sodium salt), L-glutamate (free acid), L-glutamine (free acid), and malate (free acid) were from Sigma (St. Louis, MO). Pyruvate (free acid) and succinate (free acid) were from Aldrich (Steinheim, Germany). Antimycin A was from Sigma (St. Louis, MO). HEPES was obtained from Calbiochem (La Jolla, CA). NaCl, KCl, KH_2PO_4, $NaHCO_3$, $MgSO_4$, and KOH (Suprapur) was from Merck (Darmstadt, Germany). $MgCl_2$ was from BDH Chemicals (Poole, England). Quartz-bidistilled water was used throughout.

Animals. The *ob/ob* mice are especially suited for the isolation of mitochondria from pancreatic β-cells, since they have large islets with a high proportion of β-cells (>90% [9]). The mild hyperglycemia and hyperinsulinemia of these mice are probably not due to a disturbed function of the pancreatic β-cells since there is no difference between *ob/ob* mice and their lean controls with respect to the capac-

ity to oxidize nutrient stimuli or to release insulin (10,11). In particular, the mitochondrial ATP production from *ob/ob* mice and lean controls in response to glycerol 3-phosphate was very similar (8).

Isolation of mitochondria. Adult female *ob/ob* mice, starved overnight, were used throughout. Islets were isolated by collagenase digestion of two excised pancreases. Isolated islets were gently homogenized, and a mitochondrial fraction was prepared from the homogenate after separation of cell debris and nuclei by repeated centrifugation (8). Mitochondria were purified in a buffer consisting of (mmol/l) 50 Hepes, 100 KCl, 1 EGTA, 0.05 Mg^{2+}, 1.8 ATP, and 0.5 mg/ml albumin (electrophoretically homogeneous). The pH was adjusted with KOH to 7.00 at 37°C. In the final purification step, the supernatant was replaced with 1,000 µl of the incubation medium consisting of (mmol/l) 20 HEPES, 3 KH_2PO_4, 4 carnitine, 1 EGTA, 20 NaCl, 80 KCl, 0.3 Mg^{2+}, and albumin (0.5 mg/ml, electrophoretically homogeneous). The pH was adjusted with KOH to pH 7.10 at 37°C. The mitochondrial suspension was kept on ice until used.

Incubation of mitochondria. A total of 5 µl of cold mitochondrial suspension was added to 995 µl of incubation medium prewarmed to 37°C. In a series of experiments, NaCl (20 mmol/l) was replaced by $NaHCO_3$ (20 mmol/l). This buffer was gassed for at least 1 h with O_2/CO_2 (95%/5%) before starting the incubation. During incubation, test tubes containing the modified buffer were capped after the gas space (~2 ml) was replaced with O_2/CO_2 (95%/5%). Bicarbonate-free samples were incubated in open test tubes. The incubation medium for ATP production contained the appropriate substrates, ADP (50 µmol/l) and diadenosine pentaphosphate (DAPP) (1 µmol/l). DAPP is a specific inhibitor of adenylate kinase and is routinely used in bioluminometric assays of mitochondrial ATP production (8,12). Addition of DAPP prevents extramitochondrial ATP production from ADP. Incubation was stopped by the addition of a mixture of antimycin A (0.5 µmol/l), L-luciferin (0.4 µmol/l), and luciferase (0.5 nmol/l). The samples were then rapidly cooled to room temperature, and the ATP production was determined by integrating the light produced over a period of 6 s in a Packard Tricarb scintillation spectrometer (model 3310; Packard Instrument, Meriden, CT). The spectrometer was operated out of coincidence.

Determination of [Ca^{2+}]. The free concentration of Ca^{2+} was calculated as described in detail elsewhere (8). The calculation is based on reference binding constants for Ca^{2+} and Mg^{2+} to EGTA (13). These constants were adjusted for pH (7.10) and temperature (37°C).

Data processing and standardization. Duplicate samples were carried through the entire experimental procedure. The mean of two samples was entered as one observation. To correct for inhibitory or stimulatory effects of the various experimental conditions on the luciferin/luciferase system, standards of ATP were added to parallel samples containing the complete incubation mixture but devoid of mitochondria. One experiment typically comprised about 60–80 samples resulting in 20–30 observations after subtraction of ATP standards. The amount of ATP produced during incubation was calculated from ATP standards prepared with 0.2 nmol ATP but devoid of substrates, ADP or DAPP. The quality of the mitochondrial preparation was checked by measuring adenylate kinase activity. In these experiments, mitochondria were incubated in the presence of ADP but without DAPP or substrates, and the mitochondrial ATP production by adenylate kinase activity was determined in nmol ATP after 10 min of incubation. The mitochondrial ATP production from various substrates was routinely standardized by dividing the ATP production in one experiment by the activity observed with KIC/glutamate (1 mmol/l per 10 mmol/l) in the absence of Ca^{2+}.

RESULTS

Stability of mitochondrial ATP production during incubation at 37°C. Nearly linear ATP production rates during incubation at 37°C were obtained with succinate (0.5 mmol/l). In three typical experiments, the mitochondrial ATP production averaged 0.47 ± 0.05 nmol after 10 min of incubation, 0.79 ± 0.08 nmol after 20 min, and 1.14 ± 0.11 nmol after 30 min of incubation. The effect of bicarbonate on succinate-induced ATP production was tested in parallel incubations. Bicarbonate reduced ATP production from succinate to 0.59 ± 0.05 nmol ATP during 30 min of incubation, while the ATP production rate was still stable. With all other tested substrates, the mitochondrial ATP production was stable only during the first 10 min of incubation at 37°C. Longer incubation times reduced the ATP production rate, which was almost completely arrested after 30 min. Incubation in the presence of bicarbonate did not affect the mitochondrial ATP production from KIC, KIC/glutamate, or KIC/malate. Thus in three typical experiments with KIC/glutamate (1 mmol/l/10 mmol/l), the mitochondrial ATP production averaged 0.47 ± 0.05, 0.61 ± 0.05, and 0.63 ± 0.06 nmol ATP after 10, 20, or 30 min of incubation, respectively. The corresponding ATP production measured in the presence of bicarbonate was identical. Since bicarbonate addition had no stimulatory effect on KIC-induced ATP production, it was omitted from the protocol, and all further experiments were performed in bicarbonate-free buffer. Furthermore the mitochondrial incubation time was routinely restricted to the linear ATP production phase during the first 10 min of incubation.

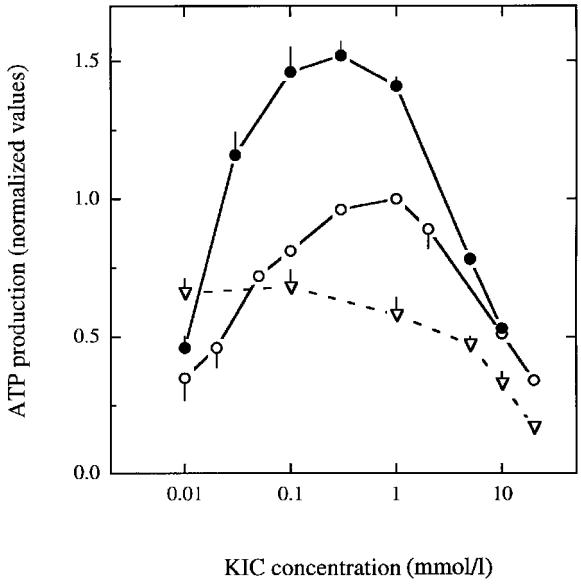

FIG. 1. Stimulation of mitochondrial ATP production by KIC. Mitochondria from pancreatic β-cells were incubated for 10 min at 37°C with malate (1 mmol/l) (▽) or with glutamate (10 mmol/l) in the absence (○) or presence (●) of Ca^{2+} (300 nmol/l). Values are normalized against the activity observed in the presence of KIC/glutamate (1 mmol/l/10 mmol/l) in the absence of Ca^{2+}. Mean values ± SE of three experiments are given.

Quality of the mitochondrial preparation. ATP production from adenylate kinase was used to estimate the day-to-day variation of the mitochondrial preparations. Adenylate kinase is located in the intermembrane space of the mitochondria; thus only structurally intact mitochondria are assayed since mitochondrial fragments are washed out during preparation. In three typical experiments, the adenylate kinase activity averaged 0.44 ± 0.02 nmol ATP after 10 min of incubation. The low standard error found for the adenylate kinase activity indicates a relatively small variation of the quality of the mitochondrial preparations. In addition, the small standard error of the mitochondrial ATP production from various substrates indicates a reproducible phosphorylation capacity in the day-to-day preparation of the mitochondria.

ATP production induced by KIC. In the presence of 1 mmol/l KIC and 10 mmol/l glutamate, mitochondria produced 0.54 ± 0.03 nmol ATP during 10 min of incubation, which corresponds to one normalized unit. Addition of 300 nmol/l Ca^{2+} increased the mitochondrial ATP production to 1.37 ± 0.04 normalized units or to 0.74 ± 0.03 nmol ATP during 10 min of incubation. Thus ~0.5 nmol ATP corresponds

TABLE 1
Substrate specificity of mitochondria isolated from pancreatic β-cells

Substrate (mmol/l)	Activity	*P* value
—	0.60 ± 0.07	
KIC (1)	0.58 ± 0.01	NS
DL-OHB (1)	0.68 ± 0.03	NS
Pal (0.001)	1.12 ± 0.08	<0.05*
Pyr (1)	1.94 ± 0.18	<0.05*
Pyr + KIC (1 + 10)	0.78 ± 0.03	NS

Data are means ± SE. Mitochondria were incubated for 10 min at 37°C in the presence of malate (1 mmol/l) and the indicated substrate additions. The concentrations were chosen to give maximal ATP production. Activities were normalized by the ATP production obtained in parallel controls with KIC/glutamate (1 mmol/l/10 mmol/l) as exclusive substrates. *P value compared with the ATP production induced by malate (1 mmol/l) alone. NS, not significant compared with the ATP production induced by malate (1 mmol/l) alone; Pal, palmitoyl-carnitine dissolved in albumin (0.5 mg/ml); Pyr, pyruvate.

to one normalized unit. This is true for all tested concentrations of KIC in the absence or presence of Ca^{2+} (Fig. 1).

Malate alone induced ATP production in β-cell mitochondria (Table 1). Palmitoyl-carnitine or pyruvate, tested at optimal substrate concentrations in the presence of malate, induced additional ATP production. By contrast, DL-OHB (1 or 10 mmol/l) or KIC (0.01–10 mmol/l) failed to induce a detectable ATP production beyond the malate-induced activity. KIC inhibited pyruvate/malate–induced ATP production (Table 1) or malate-induced ATP production in a concentration-dependent manner (Fig. 1). However, in the presence of glutamate (10 mmol/l), KIC was an efficient mitochondrial substrate (Fig. 1). In the absence of Ca^{2+}, KIC stimulated ATP production with half-maximal effect around 50 µmol/l. Maximal stimulation was observed at 1 mmol/l, whereas higher concentrations of KIC inhibited mitochondrial ATP production in a concentration-dependent manner. Addition of 20 mmol/l KIC reduced the ATP production to the basal activity observed in the absence of KIC. Like malate, glutamate (10 mmol/l) stimulated mitochondrial ATP production in the absence of additional substrates. The mitochondrial ATP production in the absence of KIC reached ~20% of the maximal activity obtained with KIC/glutamate (1 mmol/l/10 mmol/l). KIC/glutamate–induced ATP production as well as the KIC-dependent inhibition of pyruvate/malate–induced ATP production were insensitive to the addition of carnitine tested at 0, 4, or 10 mmol/l.

Ca^{2+} stimulation of mitochondrial ATP production. Ca^{2+} stimulated the ATP production induced by KIC/glutamate and reduced the concentration necessary to obtain half-maximal stimulation by KIC to <30 µmol/l (Fig. 1, closed circles). Maximal activity (1.52 ± 0.05 normalized units) was obtained with 0.3 mmol/l KIC in the presence of 300 nmol/l Ca^{2+}. The stimulatory effect of Ca^{2+} was reduced at 5 mmol/l KIC and completely disappeared in the presence of 10 mmol/l KIC. With 0.1 mmol/l KIC, half-maximal stimulation by Ca^{2+} was observed close to 200 nmol/l and maximal activity was obtained at 300 nmol/l, whereas in the presence of 500 nmol/l Ca^{2+}, only submaximal ATP production was observed (Table 2). With 500 nmol/l Ca^{2+}, the mitochondrial ATP production was variable, as indicated by the larger standard error. In the absence of Ca^{2+}, the ATP production induced by 0.5 mmol/l succinate was comparable to that induced by KIC/glutamate (0.1 mmol/l/10 mmol/l). Ca^{2+} had no stimulatory effect on the mitochondrial ATP production from succinate in a concentration range of 0–300 nmol/l, whereas 500 nmol/l Ca^{2+} strongly reduced the ATP production.

ATP production induced by leucine. Leucine stimulated mitochondrial ATP production in the presence of glutamate (10 mmol/l, data not shown). The dose-response curve for leucine was hyperbolic with a half-maximal effect at 2.0 ± 0.5 mmol/l leucine. Concentrations <0.1 mmol/l leucine did not induce a measurable ATP production above the glutamate-induced activity. Maximal stimulation (0.84 ± 0.18 normalized units) was reached at 20 mmol/l leucine.

ATP production in the presence of glutamate and glutamine. In the presence of 10 mmol/l glutamate, there were no additive effects when KIC and leucine were added at their respective maximal stimulatory concentrations (Table 3). Leucine (10 mmol/l) could not prevent the inhibition induced by 10 mmol/l KIC. With glutamine (10 mmol/l), maximal ATP production was obtained at 10 mmol/l of KIC, and no inhibition of the mitochondrial ATP production by KIC was observed. There were no additive effects of KIC and leucine when used in the presence of glutamine (10 mmol/l). In the presence of glutamate and glutamine (10 mmol/l/10 mmol/l), the mitochondrial ATP production reached a maximum at 1 mmol/l of KIC. Higher concentrations of KIC or the

TABLE 2
Effect of Ca^{2+} on succinate or KIC/glutamate–induced ATP production in isolated mitochondria of pancreatic β-cells

Ca^{2+} (nmol/l)	Succinate (0.5 mmol/l)	*P* value	KIC/glutamate (0.1–10 mmol/l)	*P* value
0	1.08 ± 0.12 (6)		0.81 ± 0.01 (6)	
100	1.06 ± 0.12 (6)	NS	1.07 ± 0.04 (8)	<0.05*
200	1.22 ± 0.13 (6)	NS	1.27 ± 0.04 (8)	<0.05*
300	1.24 ± 0.15 (6)	NS	1.48 ± 0.04 (12)	<0.05*
500	0.47 ± 0.20 (6)	<0.05*	1.19 ± 0.14 (8)	NS

Data are means ± SE (n). Activities are expressed as accumulated ATP production during 10 min of incubation at 37°C. Values are normalized with the activity observed in parallel experiments performed with KIC/glutamate (1 mmol/l/10 mmol/l) in the absence of Ca^{2+}. *P values compared with the corresponding Ca^{2+}-free controls. NS, not significant compared with the corresponding Ca^{2+}-free controls.

TABLE 3
Effect of glutamate and glutamine on mitochondrial ATP production induced by KIC or leucine

Substrate (mmol/l)	KIC (mmol/l)	Leu (mmol/l)	Activity
Glu (10)	1	10	1.02 ± 0.02
Glu (10)	10	10	0.61 ± 0.06
Gln (10)	—	—	0.22 ± 0.16
Gln (10)	1	—	0.63 ± 0.13
Gln (10)	10	—	0.78 ± 0.11
Gln (10)	1	10	0.84 ± 0.11
Gln (10)	10	10	0.83 ± 0.10
Glu/Gln (10/10)	1	—	1.06 ± 0.13
Glu/Gln (10/10)	10	—	0.99 ± 0.12
Glu/Gln (10/10)	1	10	1.10 ± 0.11
Glu/Gln (10/10)	10	10	1.01 ± 0.10

Data are means ± SE. Mitochondria were incubated for 10 min at 37°C. ATP production was normalized with the activity observed in parallel experiments performed with KIC/glutamate (1 mmol/l/10 mmol/l). Leu, leucine; Gln, glutamine; Glu, glutamate.

parallel addition of leucine did not affect the ATP production, which attained a plateau for all tested substrate combinations.

DISCUSSION

In the present study, nearly linear ATP production rates for at least 30 min were observed with succinate. This agrees well with a previous study of glycerol 3-phosphate–induced ATP production (8) and ensures that mitochondria are not subject to destruction during the incubation period at 37°C. Furthermore, stable ATP production rates demonstrate that there is no critical consumption of ADP, oxygen, or substrate during the incubation experiments. Thus the mitochondrial ATP production depends only on the variable amounts of substrates and reflects the transition between the metabolic states 2 and 3 according to Chance and Williams (13a). With isolated mitochondria, direct measurements of mitochondrial oxygen consumption as applied to isolated islets (14,15) are impossible since long preincubation times in the absence of substrates are needed for temperature equilibration of the samples, a procedure which may destroy mitochondria due to permeability transition (16).

As demonstrated here, mitochondria are not destroyed during incubation at 37°C, thus the time-dependent inhibition of mitochondrial ATP production observed for KIC-dependent ATP production may indicate a metabolic inhibition due to accumulation of acyl-CoA or NADH. It is, however, not obvious from the present experiments why succinate-induced ATP production is inhibited in the presence of bicarbonate, whereas KIC/glutamate–induced ATP production remained unaffected.

The present study was undertaken to test whether mitochondrial ATP production provides the direct metabolic link between nutrient stimulation of the pancreatic β-cell and insulin release. Several mitochondrial substrates were tested to establish their ability to induce ATP production. Specific for β-cell mitochondria appears to be the ATP production from malate alone (Table 1) (17), which is not found in rat skeletal muscle mitochondria (12). The ATP production in isolated mitochondria in the presence of malate alone indicates the presence of intramitochondrial substrate stores in the form of either acetyl-CoA or glutamate. The latter could generate α-ketoglutarate because of malate aspartate shuttle activity (18).

The close correlation between KIC-induced insulin release and KIC decarboxylation was previously attributed to the production and catabolism of acetyl-CoA (2,3,19). However, there was no net ATP production in isolated β-cell mitochondria when stimulated with KIC alone, and no KIC-dependent ATP production was observed in the presence of malate. This is not due to a defective production of acetyl-CoA in the β-cell mitochondria, since [1-^{14}C]-labeled KIC is actively decarboxylated by isolated mitochondria (data not shown) and since fatty acids or pyruvate are efficient stimulators of mitochondrial ATP production in the presence of equimolar malate concentrations. Also partial uncoupling of the β-cell mitochondria in the presence of millimolar KIC concentrations, imported by a proton symporter (20), may be excluded as a possible cause of the lack of ATP generation by KIC. Even at millimolar concentrations, KIC efficiently stimulated mitochondrial ATP production when tested in combination with glutamate and glutamine. Thus, there seems to be no direct connection between KIC decarboxylation, acetyl-CoA generation, and mitochondrial ATP production.

The insulinogenic action of KIC may, in part, be due to the generation of α-ketoglutarate after transamination with glutamate or glutamine. There is a close correlation between the concentration dependency for the production of ATP from KIC in the presence of glutamate or glutamine (Fig. 1 and Table 3) and the production of α-ketoglutarate measured in homogenates of mitochondria from pancreatic β-cells (21). Leucine was as efficient as KIC in stimulating mitochondrial ATP production, however, at much higher concentrations. Since leucine is efficiently deaminated to KIC intramitochondrially (21), the insulinogenic effect of L-leucine may be due to direct allosteric activation of glutamate dehydrogenase by leucine (22) or due to the generation of KIC.

The participation of α-ketoglutarate dehydrogenase in mitochondrial ATP production from KIC is strengthened by the observed stimulation by Ca^{2+}. An increased oxidation of labeled α-ketoglutarate in the presence of Ca^{2+} has been reported previously for islet homogenates (23). To ascribe the Ca^{2+} activation to matrix dehydrogenases, one has to exclude a possible Ca^{2+} stimulation of ATP synthase (24), which would interfere with the measurements. The effect of Ca^{2+} on the ATP production induced by succinate was used as a control. The catabolism of succinate does not involve Ca^{2+}-sensitive matrix dehydrogenases, since the malate produced from succinate is preferably exported to the cytosol (25). Since succinate-induced ATP production remained unaffected by the addition of Ca^{2+} (Table 2), any interference with the Ca^{2+}-sensitive ATP synthase on the observed Ca^{2+} stimulation could be excluded. Thus, at least in the β-cell, the Ca^{2+} activation of ATP synthase observed in mitochondria isolated from cardiomyocytes (24) does not affect mitochondrial ATP production.

In general, the stimulation of KIC-induced ATP production by Ca^{2+} resembles the recently described Ca^{2+} effects on glycerol-3 phosphate–induced ATP production in isolated β-cell mitochondria (8). Maximal activation was observed in the presence of 300 nmol/l Ca^{2+}, whereas only submaximal stim-

ulation was observed with 500 nmol/l Ca^{2+}. The reduced ATP production at 500 nmol/l Ca^{2+} is not due to a permeability transition caused by high Ca^{2+} concentrations (16). The mitochondrial ATP production was stable at least for 10 min at all tested Ca^{2+} concentrations (data not shown), indicating that the observed inhibition represents a true reduction in ATP production capacity. In contrast to KIC, the succinate-induced ATP production was strongly inhibited by the addition of 500 nmol/l Ca^{2+}. This inhibition may indicate a specific succinate-associated phenomenon. The nature of that inhibition is not obvious from the present experiments.

Compared with pyruvate/malate, KIC is not an exceptionally efficient substrate for mitochondrial ATP production. Thus, apart from ATP, there may be additional metabolic signals created by the KIC catabolism, which are of importance for the control of insulin release. When insulin release from pancreatic β-cells is stimulated by millimolar concentrations of KIC, the cytoplasmic concentration of KIC is similar to the extracellular concentration (19,26). However, mitochondrial ATP production from KIC attained a plateau at 1 mmol/l KIC. The mitochondrial KIC degradation results in the production of acetoacetate (1), which to a large extent escapes further metabolism (19,27).

Acetoacetate is a potentiator of glucose-induced insulin release both in vivo and in vitro (28–30) and induces electrical activity in the β-cell in the absence of glucose (31). The potentiation of glucose-induced release is, however, only loosely coupled to substrate oxidation. In the presence of glucose, D-β-hydroxybutyrate (D-OHB) is oxidized at the same rate as acetoacetate (27), whereas D-OHB is about twice as efficient in promoting insulin release (30). Furthermore, the potentiation of glucose-induced insulin release is not accompanied by an increased oxidation either of glucose or ketone bodies (27). Thus, despite having the capacity to oxidize ketone bodies (32), catabolism of these substrates may be ineffective and may not contribute to the generation of ATP needed for insulin secretion.

It may be that acetoacetate generated by KIC catabolism acts primarily as a nonmetabolizable signal at a membrane site separate from the ATP dependent K^+ channel. DL-OHB was used to test this hypothesis. DL-OHB is converted intramitochondrially to acetoacetate, and DL-OHB is about equally potent as acetoacetate in potentiating insulin release (30). The observed lack of DL-OHB to induce mitochondrial ATP production (Table 1) is consistent with the observation that acetoacetate is not further metabolized by the β-cell mitochondria and that it may function as a nonmetabolizable signal substance. The insulinogenic effect of KIC may be mediated by ATP generation owing to the production of α-ketoglutarate combined with the production of additional signals of which acetoacetate may be of importance.

Since KIC is as efficient as glucose to evoke a sustained insulin release (1), the proposed action of KIC implies that the β-cell is supplied with sufficient glutamate as a source for the production of α-ketoglutarate. In the absence of exogenous substrates, pancreatic β-cells sustain a high rate of respiration, which is stable for at least 2 h (15). The substrates for this endogenous respiration are mainly triglycerides and amino acids. Glutamate is by far the most abundant amino acid in the β-cell (33) amounting to 20–26 mmol/kg islet dry weight (34). Thus, the sustained endogenous respiration as well as the KIC-induced insulin release may be explained by the presence of large amounts of glutamate in the pancreatic β-cell.

The inclusion of ketone bodies in the group of nutrients that stimulate insulin release owing to an increased β-cell metabolism (27,30) is not supported by the present work. The inability of ketone bodies alone to induce insulin release both in vivo (28) and in vitro (29) is corroborated by the present results, demonstrating that DL-OHB does not stimulate mitochondrial ATP production. The insensitivity of pancreatic β-cells to ketone bodies in the absence of glucose may represent a protective mechanism against excessive insulin secretion during starvation ketosis.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Medical Research Council (12X-4756), the Swedish Diabetes Association, Svenska Sällskapet för Medicinsk Forskning, and the Sahlberg Fund.

We gratefully acknowledge Barbro Borgström for technical assistance.

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