Biochimica et Biophysica Acta, 1019 (1990) 42–50
Elsevier

BBABIO 43245

Hexose metabolism in pancreatic islets. Participation of Ca^{2+}-sensitive 2-ketoglutarate dehydrogenase in the regulation of mitochondrial function

Abdullah Sener, Joanne Rasschaert and Willy J. Malaisse

Laboratory of Experimental Medicine, Brussels Free University, Brussels (Belgium)

(Received 10 November 1989)
(Revised manuscript received 22 March 1990)

Key words: 2-Ketoglutarate dehydrogenase; Pancreatic islet; Mitochondrion; Calcium

A rise in extracellular D-glucose concentration results in a preferential and Ca^{2+}-dependent stimulation of mitochondrial oxidative events in pancreatic islet cells. The possible participation of Ca^{2+}-dependent mitochondrial dehydrogenases, especially 2-ketoglutarate dehydrogenase, in such an unusual metabolic situation was explored in intact islets, islet homogenates and isolated islet mitochondria. In intact islets exposed to a high concentration of D-glucose, the removal of extracellular Ca^{2+} impaired D-[6-^{14}C]glucose oxidation whilst failing to affect the cytosolic or mitochondrial ATP/ADP ratios. In islet homogenates, the activity of 2-ketoglutarate dehydrogenase displayed exquisite Ca^{2+}-dependency, the presence of Ca^{2+} causing a 10-fold increase in affinity for 2-ketoglutarate. In intact islet mitochondria, the oxidation of 2-[1-^{14}C]ketoglutarate also increased as a function of extramitochondrial Ca^{2+} availability. Moreover, prior stimulation of intact islets by D-glucose resulted in an increased capacity of mitochondria to oxidize 2-[1-^{14}C]ketoglutarate. The absence of extracellular Ca^{2+} during the initial stimulation of intact islets impaired but did not entirely suppress such a memory phenomenon. It is proposed that the mitochondrial accumulation of Ca^{2+} in nutrient-stimulated islets indeed accounts, in part at least, for the preferential stimulation of mitochondrial oxidative events in this fuel-sensor organ.

Introduction

The pancreatic B-cell behaves as a fuel-sensor organ, the release of insulin evoked by D-glucose or other nutrient secretagogues being causally linked to the oxidative catabolism of these nutrients with a resulting increase in ATP generation rate [1,2]. In normal islet cells, a rise in extracellular D-glucose concentration coincides with a preferential stimulation of mitochondrial oxidative events including circulation in the glycerol phosphate shuttle, pyruvate decarboxylation and oxidation of acetyl residues in the Krebs cycle [3,4]. Such a preferential stimulation is apparently linked to the activation by D-glucose (or L-leucine) of ATP-consuming functional events including the biosynthesis of proinsulin, the active pumping of Ca^{2+} and the contractile activity of the microfilamentous cell web [5,6]. Since D-glucose favours, in intact islet cells, the mitochondrial accumulation of Ca^{2+} [7,8], the feedback control of oxidative events by Ca^{2+} could conceivably reflect, in part at least, the Ca^{2+}-dependency of mitochondrial dehydrogenases, including FAD-linked glycerol-phosphate dehydrogenase, pyruvate dehydrogenase, NAD-linked isocitrate dehydrogenase and 2-ketoglutarate dehydrogenase, as already documented in several other cell types [9–16]. It was indeed recently proposed that a mitochondrial control system, involving both the movements of Ca^{2+} and generation of reducing equivalents and ATP, participates in the reciprocal coupling between metabolic and cationic events in the pancreatic B-cell [17–19]. The present study, which deals mainly with the regulation of 2-ketoglutarate dehydrogenase activity in islet mitochondria, aims at assessing the validity of this recent proposal. For the purpose of comparison, parallel experiments were also conducted in liver mitochondria, in which the Ca^{2+}-dependent activation of 2-ketoglutarate dehydrogenase was previously documented [10–14].

Materials and Methods

2-[1-^{14}C]Ketoglutarate (57–59 mCi/mmol) was purchased from either Amersham International (Amersham, U.K.) or New England Nuclear (Boston, MA,

Correspondence: W.J. Malaisse, Laboratory of Experimental Medicine, 115 Boulevard de Waterloo, B-1000 Brussels, Belgium.

U.S.A.). All solutions were prepared with ion-free H_2O (Milli-Q reagent-grade water system; Millipore, Brussels, Belgium).

Experiments with intact islets. Islets were isolated by the collagenase technique [20] from the pancreas of fed albino rats. The methods used to measure the oxidation of ^{14}C-labelled substrates [21] and the cytosolic and mitochondrial ATP and ADP content [22] in intact islets are described in the cited references.

Experiments in islet homogenates. Islets were washed twice and sonicated (3×10 s) in an imidazole-HCl buffer (50 mM, pH 7.2). Aliquots (30 μl corresponding to 40–60 islets) were brought to a final volume of 100 μl with the same buffer containing, as required, $CaCl_2$, EGTA, NAD^+, coenzyme A and 2-[1-^{14}C]ketoglutarate. The tubes containing the reaction mixture were placed in sealed counting vials, which contained 0.25 ml hyamine hydroxide (Packard, Downers Grove, IL, U.S.A.). After 30 min incubation at 37 °C, the reaction was halted by the injection of 0.1 ml HCl (0.5 M). The $^{14}CO_2$ produced during or after incubation was further collected over 60 min incubation at 20 °C. Blank values were obtained in the absence of homogenate. The generation of $^{14}CO_2$ was abolished in the absence of either NAD^+ or coenzyme A. It was constant with time (10 to 30 min) and proportional to the number of islets (20 to 120 islets). The concentration of ionized Ca^{2+} was calculated from that of EGTA and $CaCl_2$ [23].

Experiments with intact mitochondria. Pieces of liver removed from fed albino rats were minced, washed thrice and homogenized in a Hepes-KOH buffer (5.0 mM, pH 7.2) containing sucrose (60 mM), mannitol (190 mM), EGTA (0.5 mM), KCl (15 mM), $MgCl_2$ (1.0 mM) and KH_2PO_4 (3.0 mM), using a mechanical homogenizer (Braun, Melsungen, F.R.G.) with six passes at 1250 U/min. The homogenate (1.0 g wet weight liver per 2.0 ml buffer) was then brought to a total volume of 10 ml with the same buffer, and centrifuged for 15 min at $780 \times g$. The supernatant was then again centrifuged for 15 min at $6500 \times g$, the resulting pellet being resuspended in 5.0 ml of the same buffer, homogenized in Potter-Elvehjem tubes (five strokes) and then centrifuged for 15 min at $9700 \times g$ [24]. The pellet was resuspended in 1.0 ml (oxygen uptake) or 16.0 ml ($^{14}CO_2$ production) of the same buffer.

In order to isolate islet mitochondria, groups of 2500 to 3500 islets each were homogenized in Potter-Elvehjem tubes (eight strokes) in 1.5 ml of the Hepes-KOH buffer (see above). After 5 min centrifugation at $780 \times g$, an aliquot (1.0 ml) of the supernatant was removed, the remaining material being mixed with 1.0 ml of the same buffer, again homogenized (eight strokes) and centrifuged for 5 min at $780 \times g$. An aliquot (1.25 ml) of the supernatant obtained after this second centrifugation was pooled with that collected after the first centrifugation and centrifuged for 10 min at $12000 \times g$. The supernatant of this last centrifugation was removed and the pellet resuspended in 0.90 to 1.25 ml of the Hepes-KOH buffer.

All preceding manipulations were conducted at 4 °C.

In another series of experiments, groups of 300 or 600 islets each were preincubated (60 min, 37 °C) in a bicarbonate-buffer medium [20] containing bovine albumin (5 mg/ml) in the presence or absence of Ca^{2+} (1.0 mM), D-glucose (16.7 mM) and/or EGTA (0.25 mM). After the preincubation, the islets were placed in a Potter-Elvehjem tubes, washed twice with the Hepes-KOH buffer and homogenized (20 strokes) in 0.45–0.90 ml of the same buffer.

The uptake of O_2 by liver mitochondria (50 μl) was measured in the same Hepes-KOH buffer at 20 °C by polarography in a Gilson oxygraph equipped with a YSI Clark oxygen electrode (Gilson Medical Electronics, Middleton, WI, U.S.A.). Calibration was performed by the β-D-glucose/glucose oxidase method, as described elsewhere [25].

The oxidation of 2-[1-^{14}C]ketoglutarate by either liver or islet mitochondria (0.2 ml) was measured over 15 min incubation at 20 °C (liver) or 30 °C (islets), the final volume being brought to 0.4 ml with the same Hepes-KOH buffer. The incubation was halted by adding 0.1 ml of a citrate-NaOH buffer (0.4 M, pH 4.9) containing rotenone (10 μM), KCN (5.0 mM) and antimycin A (10 μM), and the $^{14}CO_2$ was recovered as described elsewhere [21].

In all these experiments, the concentration of Na^+ (used to neutralize EGTA and contributed by the disodium salt of 2-ketoglutarate) usually ranged between 1.0 to 7.0 mM.

Presentation of results. All results are expressed as the mean value ($\pm$S.E.) together with the number of individual determinations (n) or degree of freedom (d.f.). The statistical significance of differences between mean values was assessed by use of Student's t-test.

Results

Experiments in intact islets

At a high concentration of D-glucose (16.7 mM) and over 120 min incubation, the rate of D-[6-^{14}C]glucose oxidation by intact islets increased as a function of the extracellular Ca^{2+} concentration in the zero to 1.0 mM range (Fig. 1). No further increase in oxidation rate was observed, however, when the Ca^{2+} concentration was raised to 4.0 mM (data not shown). A nominal concentration of extracellular Ca^{2+} close to 0.1 mM was sufficient to cause a half-maximal oxidative response to the cation. In these experiments, the concentration of D-glucose and length of incubation were selected to ensure an optimal extent for the preferential stimulation by the hexose of mitochondrial oxidative events [5].

TABLE I

Effect of D-glucose, Ca^{2+} and cycloheximide upon the cytosolic and mitochondrial ATP/ADP ratio in intact islets

D-Glucose (mM):	2.8	16.7	2.8	16.7
Ca^{2+} (mM) [a]:	1.0	1.0	Nil	Nil
Cycloheximide (mM):	Nil	Nil	0.05	0.05
Total ATP (pmol/islet)	3.01 ±0.16 [b]	3.16 ±0.14	3.25 ±0.16	3.49 ±0.16
Total ADP (pmol/islet)	2.07 ±0.10	1.73 ±0.07	1.96 ±0.10	2.00 ±0.10
Mitochondrial ATP (pmol/islet)	1.79 ±0.11	1.55 ±0.09	1.68 ±0.11	1.73 ±0.11
Mitochondrial ADP (pmol/islet)	1.43 ±0.09	1.25 ±0.06	1.35 ±0.08	1.46 ±0.10
Mitochondrial ATP/ADP ratio	1.258±0.062	1.297±0.063	1.276±0.083	1.195±0.034
Cytosolic ATP (pmol/islet)	1.21 ±0.10	1.61 ±0.09	1.57 ±0.08	1.77 ±0.10
Cytosolic ADP (pmol/islet)	0.64 ±0.06	0.48 ±0.03	0.61 ±0.03	0.54 ±0.04
Cytosolic ATP/ADP ratio	1.950±0.080	3.402±0.156	2.699±0.191	3.427±0.242

[a] The Ca^{2+}-deprived media contained 0.25 mM EGTA but no $CaCl_2$.
[b] Mean values (±S.E.) refer in all cases to 20 individual measurements performed after 30 min incubation.

Despite the decrease in D-[6-^{14}C]glucose oxidation observed in Ca^{2+}-deprived islets exposed to a high concentration of D-glucose, the absence of extracellular Ca^{2+}, even when combined with the presence of cycloheximide to prevent the glucose-induced stimulation of protein biosynthesis [5], failed to affect significantly the cytosolic or mitochondrial ATP/ADP ratios (Table I).

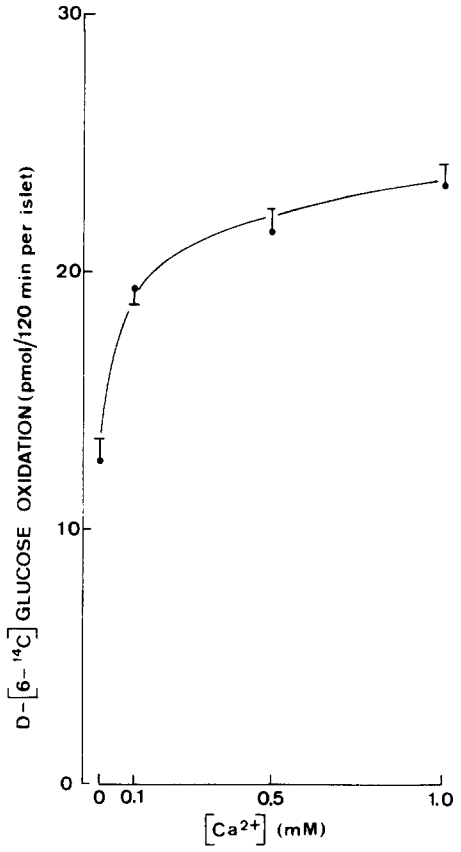

Fig. 1. Oxidation rate of D-[6-^{14}C]glucose (16.7 mM) over 120 min incubation by islets incubated at increasing Ca^{2+} concentrations. All media contained EGTA (0.5 mM) with or without the required concentration of $CaCl_2$. Mean values (±S.E.) refer to 20 individual measurements in each case.

The cytosolic, but not mitochondrial, ATP/ADP ratio was higher ($P < 0.025$ or less) in islets exposed to 16.7 mM rather than 2.8 mM D-glucose whether in the presence or absence of Ca^{2+}. At the low hexose concentration, however, the absence of Ca^{2+} and presence of cycloheximide significantly increased the cytosolic ATP/ADP ratio ($P < 0.001$).

Experiments in islet homogenates

In a series of 15 experiments, the rate of 2-[1-^{14}C]ketoglutarate decarboxylation by islet homogenates averaged 2.90 ± 0.31 pmol/min per islet, when measured in the presence of 0.5 mM Ca^{2+}, 1.0 mM NAD^+, 0.5 mM 2-ketoglutarate and 0.5 mM coenzyme A. When expressed relative to protein content, the activity of the enzyme in either normal islets or tumoral islet cells (RINm5F line) was of the same order of magnitude as that found in liver, kidney or cerebellum, whether in the absence or presence of Ca^{2+} (Table II). In islet homogenates, the K_m of the enzyme for its three substrates amounted to approx. 0.23 mM for NAD^+, 0.10 mM for 2-ketoglutarate and 0.7 μM for coenzyme A (Fig. 2). Fig. 3 documents the Ca^{2+}-dependency of the enzymatic reaction. A rise in Ca^{2+} concentration up to 16 μM caused a 10-fold increase in reaction velocity, with an apparent K_a for Ca^{2+} close to 0.7 μM. The apparent

TABLE II

Activity of 2-ketoglutarate dehydrogenase in distinct tissues

Tissue	No Ca^{2+}	Ca^{2+} (0.1 mM)
Pancreatic islets	0.87±0.21(3) [a]	8.13±0.97 (3)
RINm5F cells	1.87±0.31(4)	9.45±1.34 (4)
Liver	1.03±0.07(4)	9.88±1.14 (4)
Kidney	0.90 (1)	4.70 (1)
Cerebellum	1.29±0.18(4)	6.66±1.22 (4)

[a] All measurements were performed in the presence of 1.0 mM NAD^+, 0.5 mM 2-ketoglutarate and 0.5 mM CoA, and are expressed as pmol/min per μg protein.

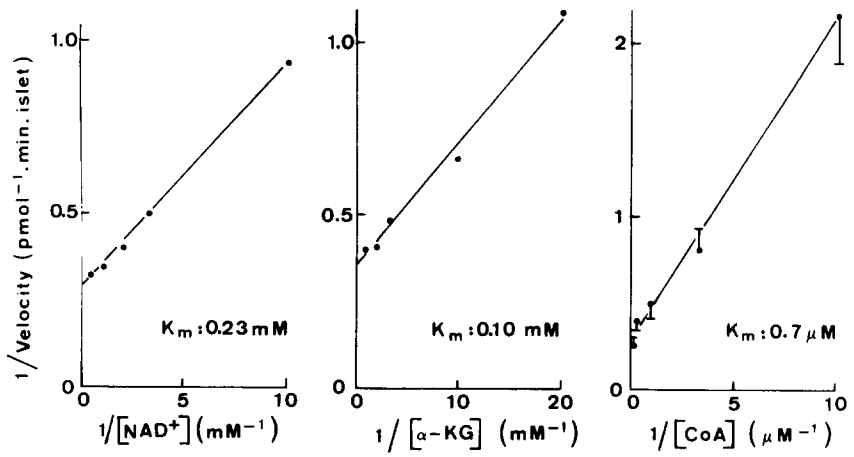

Fig. 2. Double-reciprocal plot for the activity of 2-ketoglutarate dehydrogenase in crude islet homogenates incubated for 30 min at 37°C, as a function of the concentration of NAD^+ (left panel) 2-ketoglutarate (middle panel) and coenzyme A (right panel). The concentrations of Ca^{2+} (0.5 mM), NAD^+ (1.0 mM), 2-ketoglutarate (0.5 mM) and coenzyme A (0.5 mM) were kept constant, unless otherwise indicated. In the right panel, however, the concentration of 2-ketoglutarate was decreased to 50 μM, the temperature was lowered to 20°C and the length of incubation was restricted to 15 min in order to avoid exhaustion of coenzyme A; in these experiments, the islet homogenate was first passed through a Sephadex PD-10 column in order to remove endogenous coenzyme A. Mean values (together with the range of individual variations, whenever required) are derived from triplicate measurements collected in one or two individual experiments.

K_a for Ca^{2+} was even lower (≤ 0.4 μM) when the experiments were conducted at a lower concentration of 2-ketoglutarate (0.14 mM) in the presence of thiamine pyrophosphate (0.4 mM), $MgCl_2$ (2.0 mM), EGTA (0.5 mM) and increasing concentrations of $CaCl_2$ (0.3 mM to 0.5 mM). In the absence of Ca^{2+} and presence or EGTA (0.5 mM), the K_m for 2-ketoglutarate, as measured in the presence of 1.0 mM NAD^+ and 0.5 mM coenzyme A, was increased to 1.3 mM (data not shown). Mg^{2+} (1.0 mM) exerted little effect upon reaction velocity in the presence of Ca^{2+} (1 to 10 μM) and only doubled the rate of 2-[1-^{14}C]ketoglutarate decarboxylation in the absence of Ca^{2+}. As shown in Fig. 4, the rate

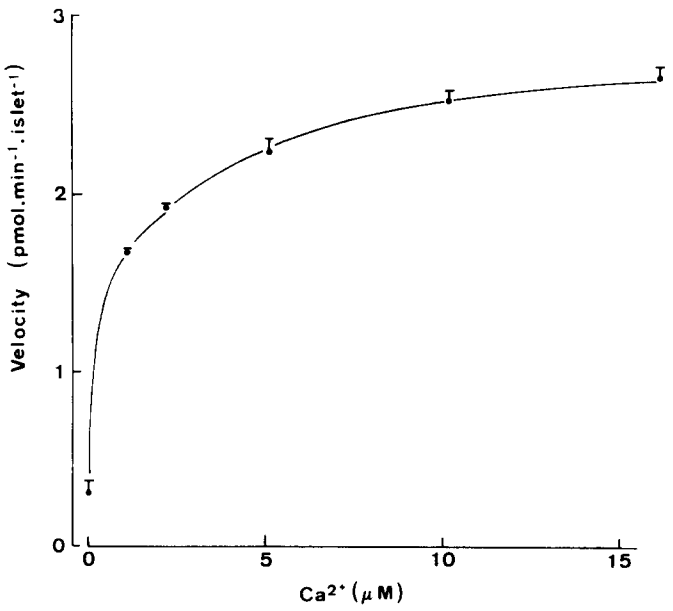

Fig. 3. Effect of increasing Ca^{2+} concentrations (EGTA 0.5 mM or EGTA and $CaCl_2$ both 10 μM, 40 μM, 0.2 mM, 0.8 mM and 2.0 mM) upon the activity of 2-ketoglutarate dehydrogenase in islet homogenates incubated at 37°C in an imidazole-HCl buffer (50 mM, pH 7.2) containing 2-[1-^{14}C]ketoglutarate (0.5 mM), NAD^+ (1.0 mM) and coenzyme A (0.5 mM). Mean values (±S.E.) are derived from triplicate measurements in each of three individual experiments.

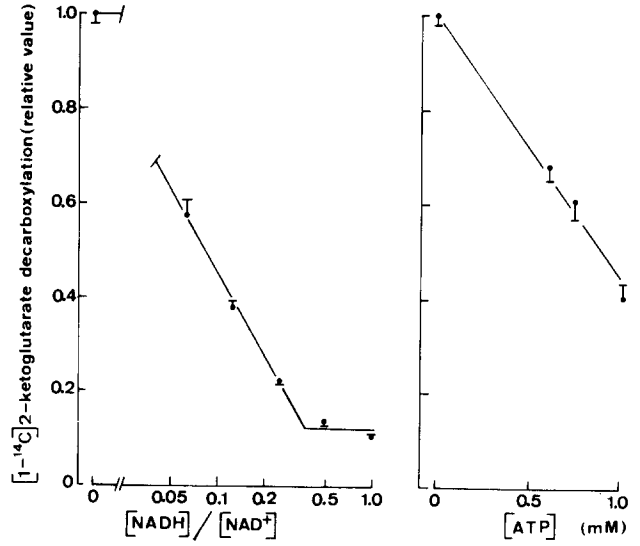

Fig. 4. Regulation of 2-ketoglutarate dehydrogenase activity in islet homogenates. Left: effect of increasing ratios of NADH/NAD^+ (logarithmic scale; see Ref. 10). Right: effect of increasing concentrations of ATP, the total concentration of ATP and ADP always amounting to 1.0 mM. All measurements were performed in the presence of 10 μM Ca^{2+}, 1.0 mM NAD^+, 0.5 mM 2-ketoglutarate and 0.5 mM coenzyme A. Mean values (±S.E.) refer to triplicate measurements collected in three separate experiments in each case, and are expressed relative to the paired control value recorded in the absence of NADH (left) or presence of 1.0 mM ADP (right).

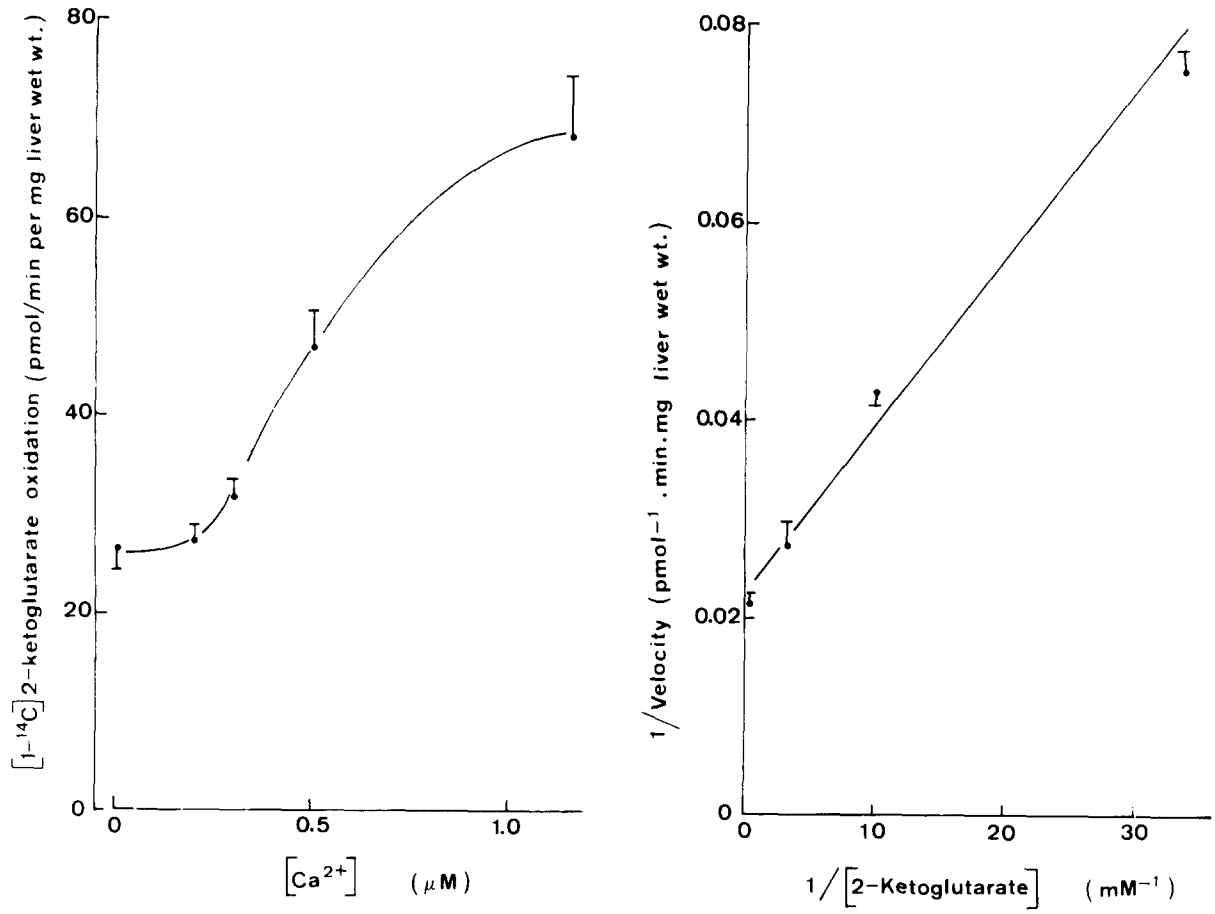

Fig. 5. Left: Ca^{2+}-dependency of the oxidation of 2-[1-^{14}C]ketoglutarate (3.0 mM) by liver mitochondria. Right: Double-reciprocal plot for the oxidation of 2-[1-^{14}C]ketoglutarate by liver mitochondria incubated at a fixed concentration of Ca^{2+} (0.5 μM) and increasing concentrations of the 2-keto acid. Mean values (±S.E.) are derived from duplicate to quadruplicate measurements collected in a series of five individual experiments in each case.

of 2-[1-^{14}C]ketoglutarate decarboxylation progressively decreased at increasing NADH/NAD^+ or ATP/ADP ratios. The influence of the NADH/NAD^+ ratio upon enzyme activity was similar in the absence of adenine nucleotide and in the concomitant presence of ATP (0.6 mM) and ADP (0.4 mM). When tested alone, ADP (1.0 mM) failed to affect significantly the reaction velocity in the presence of 10 μM Ca^{2+}. In the absence of Ca^{2+}, however, ADP (also 1.0 mM) increased 2-[1-^{14}C]ketoglutarate oxidation to 195.5 ± 12.1% (d.f. = 4; $P < 0.005$) of the paired basal value (no ADP). The incorporation of thiamine pyrophosphate in the assay medium increased the reaction velocity (Table III). As judged from the ratio in velocity at 0.14 and 0.54 mM 2-ketoglutarate, respectively, Ca^{2+} (8.0 μM) decreased the K_m for 2-ketoglutarate from 182 ± 13 to 69 ± 2 μM in the presence of exogenous thiamine pyrophosphate and from 365 ± 24 to 105 ± 8 μM in its absence ($P < 0.001$ in both cases).

TABLE III

Effect of thiamine pyrophosphate upon the activity of 2-ketoglutarate dehydrogenase in islet homogenates incubated in the presence of 1.0 mM NAD^+, 0.5 mM coenzyme A and 2.0 mM Mg^{2+}

2-Ketoglutarate (mM)	EGTA (mM)	$CaCl_2$ (mM)	Thiamine pyrophosphate	
			nil	0.2 mM
0.14	0.5	–	0.33 ± 0.01 [a]	0.71 ± 0.05
0.14	0.5	0.5	0.86 ± 0.01	1.13 ± 0.02
0.54	0.5	–	0.71 ± 0.05	1.24 ± 0.04
0.54	0.5	0.5	1.26 ± 0.09	1.50 ± 0.03

[a] Mean values (±S.E.) are derived from three measurements and expressed as pmol/min per islet.

Experiments in intact mitochondria

A first set of experiments was conducted in liver mitochondria, which are readily available in much larger amounts than islet mitochondria. In a series of eight experiments, the oxidation of 2-[1-^{14}C]ketoglutarate by liver mitochondria incubated at 20 °C in the absence of Ca^{2+} and presence of EGTA (0.5 mM) increased from 8.5 ± 0.3 to 26.7 ± 2.4 pmol/min per mg liver wet weight as the concentration of the 2-keto acid was raised from 30 μM to 3.0 mM. A rise in the extramitochondrial

TABLE IV

Effect of various agents upon 2-[1-^{14}C]ketoglutarate oxidation by liver mitochondria incubated at 20 °C

Agent (mM)	No Ca^{2+}	Ca^{2+}, 0.5 μM
Nil	44.4 ± 5.0 (6) [a]	100.0 ± 2.1 (6)
ADP (0.2)	180.1 ± 14.1 (6)	216.6 ± 12.6 (5)
Malonate (10.0)	n.d [b]	74.2 ± 2.9 (4)
Rotenone (0.01)	27.7 ± 1.1 (3)	32.5 ± 2.7 (3)

[a] Mean values (±S.E.) are derived from duplicate to quadruplicate measurements performed in 3–6 individual experiments (parentheses), and are expressed in percent of the mean value recorded in the sole presence of Ca^{2+}.

[b] Not determined.

concentration of Ca^{2+} up to 1.2 μM markedly increased such an oxidation (Fig. 5, left panel). No further increase in oxidation rate was observed in the presence of 8.0 μM Ca^{2+}, the generation of $^{14}CO_2$ progressively returning to basal value (no Ca^{2+}) when the Ca^{2+} concentration was further raised to 0.05 and 0.10 mM [11]. A concentration of extramitochondrial Ca^{2+} close to 0.5 μM was sufficient to provoke, under the present experimental conditions, a half-maximal response to the cation. At the latter concentration, the apparent K_m for 2-ketoglutarate was close to 0.08 mM (Fig. 5, right panel). In these experiments, the generation of ^{14}C-labelled amino acids, presumably L-[1-^{14}C]glutamate, represented no more than 6.9 ± 1.3% ($n = 4$) of the corresponding rate of 2-[1-^{14}C]ketoglutarate decarboxylation. As shown in Table IV, the oxidation rate of 2-[1-^{14}C]ketoglutarate (3.0 mM) by liver mitochondria was increased by ADP and inhibited by rotenone, whether in the presence or absence of Ca^{2+}. In relative terms, the effect of ADP was more marked ($P < 0.001$) in the absence of Ca^{2+} (4- to 5-fold increase) than in its presence (2-fold increase). In the presence of Ca^{2+}, malonate inhibited by 25.8 ± 3.6% (d.f = 6; $P < 0.001$) the generation of $^{14}CO_2$. These findings are consistent with the known control of 2-ketoglutarate dehydrogenase activity by its reaction product (effect of malonate), by the NADH/NAD^+ ratio (effect of rotenone and, possibly, ADP) and by ADP itself [10,26]. Thiamine pyrophosphate (0.2 mM) failed to affect significantly the oxidation of 2-[1-^{14}C]ketoglutarate (0.1 to 1.0 mM) whether in the absence or presence of Ca^{2+} (1.2 μM; data not shown).

The stimulation by Ca^{2+} of 2-[1-^{14}C]ketoglutarate oxidation coincided with a marked increase in the respiratory response of liver mitochondria to 2-ketoglutarate (Table V), even though the consumption of O_2 was assessed in the absence of exogenous ADP or uncoupling agent [11] in order to simulate the experimental conditions used in the collection of radioactive data. In the absence of Ca^{2+}, malonate (10.0 mM), which inhibited by 75% the respiratory response to succinate (also 10.0 mM), decreased the 2-ketoglutarate-induced increment in O_2 uptake to 59.7 ± 0.9% of its paired control value. Malonate, however, did not abolish the Ca^{2+}-induced stimulation of respiration recorded in the presence of 2-ketoglutarate. In the presence of succinate, instead of 2-ketoglutarate, the addition of $CaCl_2$ (final Ca^{2+} concentration: 8.0 μM) failed to stimulate O_2 uptake. Rotenone (10 μM) virtually abolished O_2 uptake under all experimental conditions, except in the presence of succinate. All these results refer to the immediate respiratory response as recorded over the first 5-6 min of exposure to each of the tested agents.

In a series of five experiments performed with mitochondria isolated from pancreatic islets, the decarboxylation rate of 2-[1-^{14}C]ketoglutarate (30 μM) averaged, in the absence of Ca^{2+} and over 15 min incubation at 30 °C, 4.09 ± 0.84 fmol/min per islet-equivalent. It represented 59.1 ± 10.1% (d.f. = 10; $P < 0.001$) of the paired value recorded in the presence of Ca^{2+} (0.5 μM). Such a percentage was similar to that recorded, within the same experiments, in liver mitochondria (64.1 ± 3.1%; d.f. = 8; $P < 0.001$). In other words, a rise in extramitochondrial Ca^{2+} concentration stimulated 2-[1-^{14}C]ketoglutarate oxidation to the same relative extent in islet and liver mitochondria. Ruthenium red (10 μM), which inhibited the uptake of ^{45}Ca by islet mitochondria [27], suppressed ($P < 0.001$) their oxidative response to Ca^{2+} (0.5 μM).

Sequential experiments in intact islets and their mitochondria

The next series of experiments aimed at investigating whether exposure of intact islets to D-glucose affects the mitochondrial rate of 2-[1-^{14}C]ketoglutarate oxidation. Groups of 300–600 islets each were incubated for 60 min at 37 °C in a salt balanced medium in the absence or presence of D-glucose (16.7 mM), washed twice in the buffer used for homogenization, and homogenized in

TABLE V

O_2 uptake by liver mitochondria incubated at 20 °C

Nutrient	Other agent	No Ca^{2+}	Ca^{2+} 8.0 μM
2-Ketoglutarate (3.0 mM)		1.48 ± 0.02 [a]	4.58 ± 0.32
2-Ketoglutarate (3.0 mM)	malonate (10.0 mM)	1.29 ± 0.01	2.33 ± 0.53
Succinate (10.0 mM)		5.77 ± 0.22	4.81 ± 0.52

[a] Mean values (±S.E.) are derived from four or more individual measurements, and expressed relative to the paired basal O_2 uptake recorded in the absence of exogenous nutrient and Ca^{2+}. Such a basal value averaged 87.2 ± 2.4 pmol of O_2 per min and mg liver wet weight.

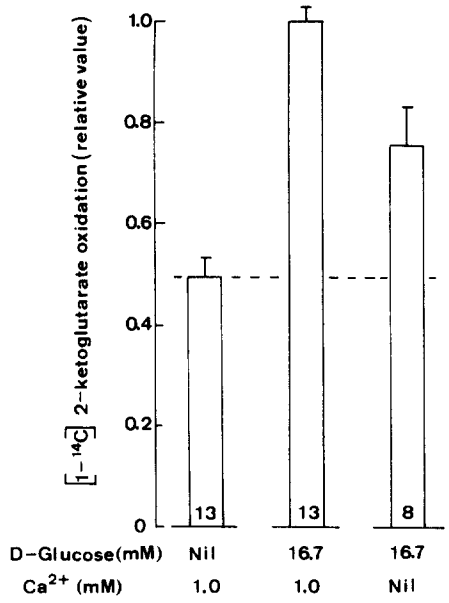

Fig. 6. Oxidation rate of 2-[1-^{14}C]ketoglutarate (30 μM) in the absence of Ca^{2+} by homogenates prepared from islets first incubated for 60 min in the presence of D-glucose and/or Ca^{2+}, as shown below each column. Mean values (±S.E.) refer to the number of measurements (each performed in duplicate) indicated at the bottom of each column, and are expressed relative to the paired control value found after exposure of the islets to both D-glucose and Ca^{2+}. Such a control value averaged 20.7 ± 3.3 fmol/min per islet.

Potter-Elvejhem tubes (20 strokes). Aliquots of the islet homogenates were then incubated for 15 min at 30 °C in the presence of 2-[1-^{14}C]ketoglutarate (30 μM) but absence of Ca^{2+} (i.e., in the presence of 0.5 mM EGTA). As illustrated in Fig. 6, in a series of five individual experiments, the rate of $^{14}CO_2$ production in the islets first deprived of D-glucose represented only 49.3 ± 4.9% (d.f. = 24; $P < 0.001$) of the paired value recorded in islets first stimulated by the hexose. This indicates that islet mitochondria keep the memory of a prior exposure of intact cells to D-glucose. Incidentally, the rate of 2-[1-^{14}C]ketoglutarate oxidation by crude homogenates prepared from islets first incubated in the absence of glucose was higher (10.73 ± 1.49 fmol/min per islet) than that recorded in mitochondria isolated from freshly collected islets (see above 4.09 ± 0.84 fmol/min per islet-equivalent), such a difference being attributable, to a large extent, to the uncomplete recovery of mitochondria in the latter procedure.

Two sets of control experiments indicated that the memory phenomenon could not be attributed to any effect of contaminating D-glucose upon the oxidation of 2-[1-^{14}C]ketoglutarate by either mitochondria or remaining intact cells in the islet homogenates. First, in liver mitochondria, D-glucose (16.7 mM) failed to affect significantly the oxidation of 2-[1-^{14}C]ketoglutarate (0.1 mM), whether in the absence or presence of Ca^{2+} (1.2 μM), the readings recorded in the presence of the hexose averaging 105.1 ± 5.4% (d.f. = 10; $P > 0.3$) of the paired control value (no D-glucose). Within the same experiments, the rate of 2-[1-^{14}C]ketoglutarate oxidation in the absence of Ca^{2+} averaged 63.3 ± 4.0% (d.f. = 10; $P < 0.001$) of that recorded in the presence of Ca^{2+}. Second, D-glucose (16.7 mM) also failed to affect the rate of $^{14}CO_2$ production by intact islets exposed at 37 °C to 2-[1-^{14}C]ketoglutarate, the ratio between experimental and control readings averaging 103.7 ± 9.6% (d.f. = 22, $P > 0.7$). Incidentally, even when the 2-keto acid was tested at a concentration of 1.0 mM, the apparent rate of 2-[1-^{14}C]ketoglutarate decarboxylation by intact islets did not exceed 26.8 ± 1.9 and 19.9 ± 1.2 fmol/min per islet ($n = 12$ in both cases), respectively in the absence of Ca^{2+} (and presence of 0.25 mM EGTA) and presence of 1.0 mM Ca^{2+} (i.e., 1.25 mM $CaCl_2$ and 0.25 mM EGTA).

It should be underlined that the memory phenomenon concerns the oxidation of 2-[1-^{14}C]ketoglutarate by intact islet mitochondria, since the sonication of isolated mitochondria virtually suppresses their capacity to generate $^{14}CO_2$ from the labelled substrate. For instance, in isolated liver mitochondria, sonication of the mitochondrial suspension decreased the oxidation rate of 2-[1-^{14}C]ketoglutarate (30 μM to 3.0 mM), as measured in the absence or presence of Ca^{2+} (1.2 μM), respectively, to 7.5 ± 0.5 and 6.0 ± 0.4% of the paired control value found in intact mitochondria.

In the last series of experiments, the possible participation of Ca^{2+} to the memory phenomenon was explored. For this purpose it was first examined, in a group of four experiments, whether a preincubation of isolated liver mitochondria for 30 min at 37 °C in the presence of Ca^{2+} increases the subsequent oxidation rate of 2-[1-^{14}C]ketoglutarate (0.1 mM) as measured during a further incubation of 15 min conducted at 20 °C in the absence of Ca^{2+}. When $CaCl_2$ (0.1 and 0.2 mM) was incorporated together with EGTA (0.5 mM) in the preincubation medium, the subsequent rate of 2-[1-^{14}C]ketoglutarate oxidation was indeed increased by 18.7 ± 4.6 and 26.2 ± 4.2%, respectively (d.f. = 12 and $P < 0.005$ in both cases), above and relative to the value found in mitochondria preincubated in the absence of Ca^{2+}. It should be noted, however, that the $^{14}CO_2$ generation rate was further increased when $CaCl_2$ (0.45 mM) was also added to the final incubation medium. Thus, during the final incubation, the ratio between basal (no Ca^{2+}) and Ca^{2+}-stimulated oxidation rates averaged 53.1 ± 5.0 and 60.2 ± 3.6% (d.f. = 12 in both cases) in the mitochondria preincubated in the presence of 0.1 and 0.2 mM $CaCl_2$, respectively, as compared to 44.4 ± 3.9% (d.f. = 6) in the mitochondria preincubated in the absence of the cation. The relative extent of stimulation by Ca^{2+} during the final incubation was thus inversely related to the $CaCl_2$ content of the preincubation medium. In the next protocol, intact

islets were first incubated for 60 min at 37 °C in the presence of D-glucose (16.7 mM) either at normal Ca^{2+} concentration (1.0 mM) or in the absence of $CaCl_2$ and presence of EGTA (0.25 mM). In the latter case, the subsequent rate of 2-[1-^{14}C]ketoglutarate oxidation by islet homogenates, as measured in the absence of Ca^{2+}, only represented 75.4 ± 9.0% (d.f. = 14; $P < 0.02$) of the paired control value (Fig. 6). This indicates that the absence of extracellular Ca^{2+} during the initial incubation impairs but does not totally suppress the glucose-induced memory phenomenon. Indeed, the $^{14}CO_2$ production rate by homogenates derived from islets first exposed to D-glucose in the absence of Ca^{2+} remained higher ($P < 0.02$) than that found in islets first incubated in the absence of the hexose at normal Ca^{2+} concentration (Fig. 6). Incidentally, the incubation of islets in Ca^{2+}-deprived media also slightly decreased the subsequent rate of 2-[1-^{14}C]ketoglutarate oxidation when the initial incubation medium contained no exogenous nutrient, the experimental (no Ca^{2+}) to control (1.0 mM Ca^{2+}) ratio averaging 74.6 ± 7.8% (d.f. = 4). Taken as a whole, the present findings suggest, therefore, that the glucose-induced memory phenomenon may be attributable, in part but not exclusively, to the increased influx of Ca^{2+} provoked by the hexose in intact islets incubated at normal extracellular Ca^{2+} concentration [28]. Along the same line of thinking, it should be underlined that the memory phenomenon did not coincide with a full activation of 2-ketoglutarate dehydrogenase by mitochondrial Ca^{2+}. Indeed, a rise in extramitochondrial Ca^{2+} concentration to 0.5 μM invariably augmented 2-[1-^{14}C]ketoglutarate oxidation by the islet homogenates, whether the islets had been first incubated in the absence or presence of D-glucose and/or Ca^{2+}. In these experiments, the ratio of basal (no Ca^{2+}) to Ca^{2+}-stimulated $^{14}CO_2$ production by the islet homogenates averaged 67.9 ± 5.8% (d.f. = 14; $P < 0.001$) when the islets were first incubated either in the presence of Ca^{2+} (1.0 mM) but absence of exogenous nutrient or absence of Ca^{2+} but presence of D-glucose (16.7 mM), and 65.4 ± 5.6% (d.f. = 14; $P < 0.001$) when the islets were first incubated in the presence of both the cation and hexose.

Discussion

The present results indicate that, in sonicated islets, as in other cell types, 2-ketoglutarate dehydrogenase displays exquisite Ca^{2+}-sensitivity, a rise in Ca^{2+} concentration coinciding with a 10- to 14-fold increase in affinity for 2-ketoglutarate. The activity of 2-ketoglutarate dehydrogenase is also regulated by the NADH/NAD^+ and ATP/ADP ratios. The Ca^{2+}-dependency of the enzyme was further documented in intact islet mitochondria incubated in the absence or presence of Ca^{2+}. The specific activity of the enzyme was not vastly different in islet and other tissues. Likewise, relative to tissue wet weight, the oxidation rate of 2-[1-^{14}C]ketoglutarate (30 μM), in the absence of Ca^{2+}, was of the same order of magnitude in liver mitochondria and crude islet homogenates.

Prior stimulation of intact islets by D-glucose (16.7 mM) resulted in an increased capacity of mitochondria to oxidize 2-[1-^{14}C]ketoglutarate, as measured in the absence of Ca^{2+}. In these experiments, the mitochondria were not isolated from other subcellular fractions in order to minimize the delay between the initial incubation of intact islets and the final measurement of 2-[1-^{14}C]ketoglutarate oxidation. The memory phenomenon could conceivably result, in part at least, from the stimulation by the hexose of Ca^{2+} influx into the B-cell, this coinciding with an increase in mitochondrial Ca^{2+} content [7,8]. The memory phenomenon was indeed impaired when the islets were first incubated in the presence of D-glucose, but absence of extracellular Ca^{2+}. A decrease in extracellular Ca^{2+} availability also coincided with a concentration-related impairment of D-[6-^{14}C]glucose oxidation, provided that the islets were exposed to a high concentration of the hexose [5].

Taken as a whole, these results suggest that, in islet cells, as in other cell types [9–12], the mitochondrial accumulation of Ca^{2+} associated with cell activation may account for an increased oxidation of substrates in these organelles and, hence, may help to meet the increased energy demand of the activated cells. Further work is in progress to extend this concept to other islet mitochondrial dehydrogenases. Available information indeed supports the view that D-glucose, in the presence of extracellular Ca^{2+}, may favour the activity of glycerol-phosphate dehydrogenase [3,29], pyruvate dehydrogenase [3] and isocitrate dehydrogenase [5] in pancreatic islets.

Although the analogy between islet cells and other cell types cannot be ignored, two specific features of the endocrine pancreas merit emphasis. First, at variance with the situation found in other tissues, the mitochondrial accumulation of Ca^{2+} upon cell activation might not be solely attributable to an increase in cytosolic Ca^{2+} activity as resulting, for instance, from the gating of voltage-sensitive Ca^{2+} channels [30]. Thus, the mitochondrial memory phenomenon, documented in islets first exposed to D-glucose, was not completely supressed when the initial incubation was conducted in the absence of extracellular Ca^{2+}. It is conceivable, therefore, that some other factor(s) participate(s) in the memory phenomenon. For instance, even in the absence of extracellular Ca^{2+}, D-glucose could favour the mitochondrial accumulation of Ca^{2+} by increasing the generation rate of ATP and/or reducing equivalents. The ATP availability may indeed regulate Ca^{2+} uptake by mitochondria [31]. The present results indicate that glucose increases the cytosolic ATP/ADP ratio whether the islets are incubated in the presence or absence of Ca^{2+}.

The removal of extracellular Ca^{2+} even minimized the fall in cytosolic ATP concentration otherwise recorded in islets deprived of exogenous nutrient. Likewise, the induction of a more reduced mitochondrial state in glucose-stimulated islets could also favour Ca^{2+} accumulation by decreasing the efflux of the cation from these organelles [32]. It should be stressed, however, that, to our knowledge, the mitochondrial redox state has never been measured in islet cells. Anyhow, the view that D-glucose favours the sequestration of Ca^{2+} into mitochondria (and possibly other organelles), even in the absence of extracellular Ca^{2+}, is compatible with the fact that the hexose causes a rapid and sustained decrease in ^{45}Ca fractional outflow rate from prelabelled islets exposed to Ca^{2+}-deprived perifusates [33,34].

The second difference between islet cells and other cell types consists in the fact that, in the former cells, the process of cell activation by D-glucose already involves a cause-to-effect relationship between early metabolic and more distal cationic events, instead of being solely attributable to the occupancy of specific membrane receptors by a suitable neurotransmitter or hormone acting as first messenger. Thus, it is currently believed that, in the pancreatic B-cell, the glucose-induced acceleration of ATP generation and coinciding increase in cytosolic ATP/ADP ratio represents the major coupling process responsible for the closing of ATP-responsive K^+ channels [22]. The resulting decrease in K^+ conductance may then lead to depolarization of the plasma membrane and gating of voltage-sensitive Ca^{2+} channels. If so, the activation of mitochondrial dehydrogenases, as revealed in the present study, could be looked upon as a process allowing for further amplification of the functional response to exogenous nutrients in endocrine pancreatic cells.

In conclusion, therefore, the preferential stimulation of oxidative mitochondrial processes and the activation by Ca^{2+} of key mitochondrial dehydrogenases might well represent tightly interrelated events of critical importance in the functional response of islet cells to nutrient secretagogues.

Acknowledgements

We wish to thank J. Schoonheydt and M. Urbain for technical assistance and C. Demesmaeker for secretarial help. This work was supported by grants from the Belgian Foundation for Scientific Medical Research and Belgian Ministry of Scientific Policy. J.R. is a predoctoral fellow of the Belgian Institute for Scientific Research in Industry and Agriculture.

References

1 Malaisse, W.J., Sener, A., Herchuelz, A. and Hutton, J.C. (1979) Metabolism 28, 373–386.
2 Malaisse, W.J. (1983) Diab. Metab. 9, 313–320.
3 Sener, A. and Malaisse, W.J. (1987) Biochem. J. 246, 89–95.
4 Sener, A., Rasschaert, J., Zähner, D. and Malaisse, W.J. (1988) Int. J. Biochem. 20, 595–598.
5 Malaisse, W.J. and Sener, A. (1988) Biochim. Biophys. Acta 971, 246–254.
6 Sener, A., Malaisse-Lagae, F. and Malaisse, W.J. (1982) Biochem. J. 202, 309–316.
7 Andersson, T., Betsholtz, C. and Hellman, B. (1982) Biomed. Res. 3, 29–36.
8 Andersson, T., Berggren, P.-O., Gylfe, E. and Hellman, B. (1982) Acta Physiol. Scand. 114, 235–241.
9 Denton, R.M., McCormack, J.G. and Edgell, N.J. (1980) Biochem. J. 190, 107–117.
10 Hansford, R.G. (1985) Rev. Physiol. Biochem. Pharmacol. 102, 1–72.
11 McCormack, J.G. (1985) Biochem. J. 231, 581–595.
12 Denton, R.M. and McCormack, J.G. (1986) Cell Calcium 7, 377–386.
13 McCormack, J.G. (1985) Biochem. J. 231, 597–608.
14 Johnston, J.D. and Brand, M.D. (1987) Biochem. J. 245, 217–222.
15 McCormack, J.G. and Denton, R.M. (1984) Biochem. J. 218, 235–247.
16 Lawlis, V.B. and Roche, T.E. (1981) Biochemistry 20, 2519–2524.
17 Malaisse, W.J., Blachier, F., Pochet, R., Manuel y Keenoy, B. and Sener, A. (1989) in Calcium Binding Proteins in Normal and Transformed Cells (Lawson, D.E.M. and Pochet, R., eds.), pp. 127–133, Plenum, New York.
18 Malaisse, W.J. (1989) in Proceedings 5th European Symposium on Metabolism, Elsevier, Amsterdam, in press.
19 Malaisse, W.J., Malaisse-Lagae, F., Rasschaert, J., Zähner, D., Sener, A. and Van Schaftingen, E. (1990) Biochem. Soc. Trans. 18, 107–108.
20 Malaisse-Lagae, F. and Malaisse, W.J. (1984) in Methods in Diabetes Research (Larner, R. and Pohl, S.L., eds.), pp. 147–152, John Wiley & Sons, New York.
21 Carpinelli, A.R., Sener, A., Herchuelz, A. and Malaisse, W.J. (1980) Metabolism 29, 540–545.
22 Malaisse, W.J. and Sener, A. (1987) Biochim. Biophys. Acta 927, 190–195.
23 Hurley, T.W., Becker, J.K. and Martinez, J.R. (1984) J. Biol. Chem. 259, 7061–7066.
24 Pedersen, P.L., Greenawalt, J.W., Reynafarje, B., Hullihen, J., Decker, G.L., Soper, J.W. and Bustamente, E. (1978) Methods Cell Biol. 20, 411–481.
25 Blachier, F., Sener, A. and Malaisse, W.J. (1987) Biochim. Biophys. Acta 921, 494–501.
26 Garland, P.B. (1974) in Methods of Enzymatic Analysis, 2nd Edn. (Bergmeyer, H.U., ed.), Vol. 4, pp. 1981–1987, Academic Press, New York.
27 Manuel y Keenoy, B., Levitsky, D.O., Sener, A. and Malaisse, W.J. (1990) Diabetes Res., in press.
28 Wollheim, C.B. and Sharp, G.W.G. (1981) Physiol. Rev. 61, 914–973.
29 McDonald, M.J. (1982) Horm. Metab. Res. 14, 678–679.
30 Deleers, M., Mahy, M. and Malaisse, W.J. (1985) Biochem. Int. 10, 97–103.
31 Nicholls, D. and Akerman, K. (1982) Biochim. Biophys. Acta 683, 57–88.
32 Lehninger, A.L., Reynafarje, B., Vercesi, A. and Teu, W.P. (1978) Ann. N.Y. Acad. Sci. 307, 160–176.
33 Malaisse, W.J., Brisson, G.R. and Baird, L.E. (1973) Am. J. Physiol. 224, 389–394.
34 Scholler, Y., De Maertelaer, V. and Malaisse, W.J. (1984) Biophys. J. 46, 439–446.