Regulation of pancreatic β -cell mitochondrial metabolism: influence of Ca^{2+} , substrate and ADP

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To gain insight into the regulation of pancreatic β -cell mitochondrial metabolism, the direct effects on respiration of different mitochondrial substrates, variations in the ATP/ADP ratio and free Ca^{2+} were examined using isolated mitochondria and permeabilized clonal pancreatic β -cells (HIT). Respiration from pyruvate was high and not influenced by Ca^{2+} in State 3 or under various redox states and fixed values of the ATP/ADP ratio; nevertheless, high Ca^{2+} elevated pyridine nucleotide fluorescence, indicating activation of pyruvate dehydrogenase by Ca^{2+} . Furthermore, in the presence of pyruvate, elevated Ca^{2+} stimulated CO_2 production from pyruvate, increased citrate production and efflux from the mitochondria and inhibited CO_2 production from palmitate. The latter observation suggests that β -cell fatty acid oxidation is not regulated exclusively by malonyl-CoA but also by the mitochondrial redox state. α -Glycerophosphate (α -GP) oxidation was Ca^{2+} -dependent with a half-maximal rate observed at around 300 nM Ca^{2+} . We have recently demonstrated that

increases in respiration precede increases in Ca^{2+} in glucose-stimulated clonal pancreatic β -cells (HIT), indicating that Ca^{2+} is not responsible for the initial stimulation of respiration [Civelek, Deeney, Kubik, Schultz, Tornheim and Corkey (1996) *Biochem. J.* **315**, 1015–1019]. It is suggested that respiration is stimulated by increased substrate (α -GP and pyruvate) supply together with oscillatory increases in ADP [Nilsson, Schultz, Berggren, Corkey and Tornheim (1996) *Biochem. J.* **314**, 91–94]. The rise in Ca^{2+} , which in itself may not significantly increase net respiration, could have the important functions of (1) activating the α -GP shuttle, to maintain an oxidized cytosol and high glycolytic flux; (2) activating pyruvate dehydrogenase, and indirectly pyruvate carboxylase, to sustain production of citrate and hence the putative signal coupling factors, malonyl-CoA and acyl-CoA; and (3) increasing mitochondrial redox state to implement the switch from fatty acid to pyruvate oxidation.

INTRODUCTION

Stimulation of insulin secretion from pancreatic islets by glucose and other fuels is associated with increased respiration [1–3]. The stimulus–secretion coupling mechanism involves an increase in intracellular free Ca^{2+} [4]. The intramitochondrial enzymes pyruvate dehydrogenase (PDH), 2-oxoglutarate (2-OG) dehydrogenase and NAD-linked isocitrate dehydrogenase are all Ca^{2+} -sensitive [5,6]. We have shown previously that PDH is converted from its inactive phosphorylated form into its active dephosphorylated form when islets are stimulated with glucose or high extracellular Ca^{2+} [7], in keeping with the Ca^{2+} sensitivity of PDH-P phosphatase [5,6]. Furthermore, islet mitochondria contain a high activity of the flavin-linked α -glycerophosphate (α -GP) dehydrogenase, which is on the cytosolic side of the inner mitochondrial membrane and is also activated by Ca^{2+} [8–13]. Therefore, it has been suggested that the increased respiration seen in glucose-stimulated islets is a consequence of stimulation of these dehydrogenases by the elevated Ca^{2+} , in order to cover the rising energy demand of secretion [5,7,13,14]. The question of whether increased Ca^{2+} in the β -cell could account for the fuel-induced increases in respiration has not been directly evaluated. Increases in the mitochondrial substrates α -GP [15] and pyruvate [16] also occur upon glucose stimulation and they could also increase respiration by increasing substrate supply. A major quandary in understanding fuel-mediated increases in respiration

is the well-established finding that there is an increase in the average ATP/ADP ratio [17–19] which would be expected to inhibit respiration.

The studies presented here directly examined the effects of different mitochondrial substrates, variations in the ATP/ADP ratio and Ca^{2+} on respiration, using isolated mitochondria and permeabilized clonal pancreatic β -cells (HIT) with the goal of identifying factors responsible for the increase in respiration induced by stimulatory concentrations of glucose. Our data document high rates of respiration from pyruvate and the expected inhibition in response to increases in the ATP/ADP ratio. They confirm that the mitochondrial α -GP dehydrogenase is very active, sensitive to the changes in α -GP occurring in response to glucose and regulated by physiological concentrations of Ca^{2+} . However, variations in Ca^{2+} did not appear to influence net respiration from pyruvate, even though increases in Ca^{2+} increased the mitochondrial redox state, increased citrate production from pyruvate and decreased fatty acid oxidation in the presence of pyruvate.

EXPERIMENTAL

Growth and incubation of cells, mitochondria and islets

Clonal pancreatic β -cells (HIT-T 15) were cultured in RPMI 1640 medium supplemented with 50 units/ml penicillin, 50 $\mu\text{g}/\text{ml}$

Abbreviations used: α -GP, α -glycerophosphate; 2-OG, 2-oxoglutarate; PDH, pyruvate dehydrogenase; redox state, pyridine nucleotide oxidation state.

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streptomycin and 10% (v/v) fetal-calf serum, used between passages 64 and 80, harvested with PBS containing 0.02% EDTA, and washed in Hanks' Ca^{2+} - Mg^{2+} -free buffer, pH 7.4 [20]. Cells were incubated in either a fixed ATP/ADP ratio buffer [21] containing 100 mM KCl, 22 mM NaCl, 5 mM KHCO_3 , 20 mM HEPES, pH 7.4, 1 mM MgCl_2 , 6 mM KH_2PO_4 , 4 mM MgATP, creatine plus creatine phosphate (to total 12 mM) and 50 $\mu\text{g}/\text{ml}$ creatine phosphokinase, or an ADP-regenerating system (identical to the fixed ratio system but without creatine phosphate). The values used for the ATP/ADP ratio are based on free Mg:ADP calculated from the creatine phosphokinase equilibrium constant and the measured concentrations of creatine, creatine phosphate and ATP [21]. Malate (0.5 mM) was added during respiration with pyruvate or glutamate to maintain the mitochondrial pH gradient by permitting exchange of phosphate and H^+ ions [22]. The concentration of pyruvate (0.1 mM) used for most of the studies was selected to stimulate respiration without activating PDH [22]. Cells were added at a final concentration of 1–2.5 mg of protein/ml. Saponin (60–80 $\mu\text{g}/\text{ml}$) was added to selectively permeabilize the plasma membrane of cells in suspension. The precise concentration used was the minimum amount required to permeabilize the plasma membrane within 5 min without altering the Ca^{2+} set point maintained by the permeabilized cells. Data presented in the Figures were from pair-matched experiments, since variations of about 2-fold in maximum respiratory rates were observed in cells from different passages. Islets were prepared and incubated as described by Longo et al. [23]. Mitochondria were isolated from tumours grown in NEDH rats as described previously [24]. States 3 and 4 respiration were determined in the presence of buffer and substrate with and without ADP, respectively. Experiments were performed at 30 °C where many processes take place more slowly [17], including cell deterioration, but insulin secretion occurs [25].

Ca^{2+} measurements

Ca^{2+} values in the presence of EGTA were determined using a Ca^{2+} electrode calibrated with Ca^{2+} standards from World Precision Instruments (Sarasota, FL, U.S.A.).

Oxygen consumption

O_2 consumption was measured using a Clark-type electrode with an amplifier designed and built by the Bio-Instrumentation Group of the University of Pennsylvania in a stirred, water-jacketed, closed chamber. At the high sensitivity required for permeabilized-cell O_2 measurement, the electrode itself consumed considerable O_2 . An illustration of the response obtained in permeabilized cells is shown in Figure 1. The low rate of O_2 consumption, observed in the presence of the buffer alone and due to O_2 use by the electrode, was subtracted from the subsequent rates. Further increases in O_2 consumption were observed upon sequential addition of cells (in the presence of ADP), α -GP and Ca^{2+} . These corrected rates were converted into nmol of O_2 used based on a concentration of dissolved O_2 in the buffer of 225 μM [26]. The permeabilized cells were sensitive to the ambient ATP/ADP ratio which was fixed as described previously [21]. The fixed ratios are maintained in the presence of endogenous ATPases because of the high concentrations of creatine and creatine phosphate in the buffering system. Cells responded with decreasing rates of respiration as the ratio was increased over the physiological range of values, giving rates of 7, 4.5 and 1.7 nmol/min per mg of protein at ATP/ADP ratios of 3, 15 and 150, respectively. Thus, permeabilized cells are able to respond to substrate and are sensitive to adenine nucleotide

Figure 1 Measurement of O_2 consumption in permeabilized β -cells (HIT)

The technique used to measure O_2 consumption in cells (2–2.5 mg protein/ml) permeabilized (60–80 $\mu\text{g}/\text{ml}$ saponin) in media containing EGTA (0.1 mM) and a fixed ATP/ADP ratio of 3 is illustrated: Cells, α -GP (1 mM) and Ca^{2+} were added at the times indicated by the arrows. The Ca^{2+} concentration was increased to approx. 1 μM as determined using a Ca^{2+} electrode. The basal rate of O_2 consumption, before addition of cells, represents O_2 consumption by the electrode. Extrapolated rates of respiration, used for calculations are indicated by broken lines.

changes and, consequently, provide a useful preparation for studying mitochondrial function within a relatively normal architectural environment.

CO_2 production

$^{14}\text{CO}_2$ evolution was measured from permeabilized β -cells incubated in the presence of [$1\text{-}^{14}\text{C}$]pyruvate or prelabelled overnight in media containing 1 μM [$\text{U-}^{14}\text{C}$]palmitate and incubated at a final pyruvate concentration of 0.1 mM. Following incubation in sealed flasks for 0 or 30 min, the reaction was stopped by the addition of perchloric acid [27] and evolved $^{14}\text{CO}_2$ trapped in 200 μl of phenethylamine (contained in a suspended well in the sealed flask) by incubation for 1 h. Radioactivity in the well containing trapped $^{14}\text{CO}_2$ was counted in a scintillation counter. Correction was made for spontaneous decarboxylation by subtracting counts obtained at 0 min or following incubation in buffer without cells for 0 and 30 min. Cells prelabelled with palmitate were washed three times and incubated without added labelled or unlabelled palmitate. The total amount of radioactive palmitate incorporated into chloroform/methanol (2:1, v/v)-extractable cell lipid was 170 pmol/mg of protein and results are expressed as pmol of CO_2 derived from this pool.

Pyridine nucleotide fluorescence

Pyridine nucleotide fluorescence was measured using an Hitachi Model F 2000 spectrofluorimeter, equipped with a temperature-controlled, stirring cuvette holder, set at wavelengths of 340 nm excitation and 460 nm emission.

Assays and materials

α -GP levels (from about 100 islets) and citrate production (from permeabilized cells) were measured in neutralized perchloric acid extracts [28]. RPMI 1640 medium, penicillin and streptomycin were from Gibco. Fetal-calf serum was obtained from Hyclone Laboratories. Biochemicals were from Sigma or Boehringer Mannheim.

RESULTS

Respiration from different substrates, in mitochondria isolated from a glucose-responsive β -cell tumour, showed rapid rates of State 3 respiration (defined as excess O_2 , phosphate and substrate plus ADP) in the presence of the flavin-linked substrates, succinate and α -GP, and the NAD-linked substrates, 2-OG, glutamate plus malate and pyruvate plus malate (Table 1). Addition of malate did not influence respiration from the 2-OG at the concentrations used under the present conditions (results not shown). Chelation of the contaminating Ca^{2+} with EGTA markedly inhibited respiration from α -GP (Table 1) but not from the other substrates (results not shown). Respiratory control ratios (State 3/State 4) of 5 to 8 and ADP/O ratios of 2 to 3 were obtained for the NAD-linked substrates, indicating healthy, well-coupled mitochondria. The high rate of respiration using α -GP as a substrate is consistent with reports of high levels of α -GP dehydrogenase enzyme activity in β -cells [8,11].

To study further the relative contributions of Ca^{2+} , substrate and ADP in the regulation of respiration and mitochondrial metabolism, we used a permeabilized clonal β -cell line (HIT) where the time-consuming and potentially damaging mitochondrial purification process was not necessary. This model has the advantage of maintaining a physiological low free Ca^{2+} concentration, of preserving mitochondria in their normal intracellular location relative to other organelles, and of being available for study within seconds of disrupting the plasma membrane. The permeabilized cell preparation has the disadvantage of continuously converting ATP into ADP due to the high activity of cellular ATPases present in the plasma membrane and endoplasmic reticulum. This does not affect the assessment of maximal State 3 rates of respiration but prevents accurate measurement of respiratory control ratios, since added ADP is continuously recycled. Respiratory control was assessed using the simpler isolated mitochondria preparation (Table 1). Like the isolated mitochondria, respiration in permeabilized clonal β -cells (HIT) showed rapid rates of State 3 respiration in the presence of an ADP-regenerating system (creatine + ATP $\rightarrow$ creatine phosphate + ADP) and either succinate, α -GP, 2-OG, or pyruvate plus malate (Table 2). Unlike the isolated mitochondria, in the

Table 2 Oxygen consumption by permeabilized clonal pancreatic β -cells (HIT)

Concentrations of substrates were: α -GP and succinate, 5 mM; 2-OG, 0.25 mM or 5 mM; malate, 0.5 mM (with pyruvate as substrate); and pyruvate, 0.05, 0.1 mM or 5 mM, as indicated. Cells were permeabilized by addition of 60–80 μ g/ml saponin in a buffer consisting of 100 mM KCl, 20 mM Hepes, 5 mM $KHCO_3$, 1 mM $MgCl_2$, 6 mM KH_2PO_4 , 5 mM NaCl, 5 mM creatine, 0.5 mg/ml creatine phosphokinase, 1 mM ADP (State 3) at 80 nM or 1 μ M Ca^{2+} (where indicated), pH 7.4. Values shown are means $\pm$ S.E.M. of 3–6 experiments from separate preparations and represent increments above the basal value of 3.5 ± 1.2 nmol/min per mg of protein.

Additions	Oxygen consumption (nmol/min per mg of protein)
α -GP	5.7 ± 0.1
Succinate	11.3 ± 1.2
2-OG (0.25 mM)	1.8 ± 0.5
2-OG (5.0 mM)	3.3 ± 0.4
Pyruvate (0.05 mM)	0.72 ± 0.1
Pyruvate (0.1 mM)	1.8 ± 0.1
Pyruvate (0.1 mM + 1 μ M Ca^{2+})	1.8 ± 0.05
Pyruvate (5.0 mM)	3.8 ± 0.6

Figure 2 Concentration-dependence of α -GP- (A and B) and Ca^{2+} - (C and D) stimulated O_2 consumption in permeabilized β -cells

O_2 consumption was measured as described in Figure 1. In (A) the Ca^{2+} concentration was either 1 μ M, or 100 nM in the presence of 0.1 mM EGTA as determined using a Ca^{2+} electrode. (B) shows a double reciprocal plot of the data in (A). Linear regression analyses gave lines with the formulae $y = 0.06 + 0.16x$ (upper trace, B) and $y = 0.06 + 0.06x$ (lower trace, B) with r values of 0.993 and 0.969 in the presence of EGTA or added Ca^{2+} , respectively. Calculated apparent half-maximal values for α -GP were 2.5 and 1 mM in the presence of EGTA or added Ca^{2+} , respectively, with an apparent V_{max} value of 17 nmol/min per mg of protein, in the presence or absence of Ca^{2+} . In (C) Ca^{2+} was varied and the α -GP concentration was 1 mM. (D) shows a double reciprocal plot of the data in (C). Linear regression analysis of these data gave a line with the formula $y = 0.05 + 14x$ and an r value of 0.975. The calculated apparent half-maximal value for Ca^{2+} activation was 315 nM. Data shown in (A) and (C) are means $\pm$ S.E.M. of three experiments from separate preparations.

absence of added substrate, permeabilized cells exhibited basal rates of respiration (3.5 ± 1.2 nmol/min per mg of protein) higher than the increment observed with NAD-linked substrates, presumably due largely to endogenous fatty acids.

The high rate of respiration stimulated by α -GP was concentration-dependent (Figure 2A). The α -GP concentration de-

Table 1 Oxygen consumption by isolated rat insulinoma mitochondria

Substrate concentrations were 5 mM except where stated. Mitochondria were isolated from tumours grown in NEDH rats as described in ref. [23]. Respiration was measured with a Clark-type oxygen electrode using a water-jacketed Perspex chamber maintained at 30 °C. The incubation medium consisted of 115 mM KCl, 5 mM KH_2PO_4 , 20 mM Hepes, 1 mM $MgCl_2$, 0.5 mg/ml of BSA, pH 7.2. Mitochondria were present at a concentration of 0.5 mg of protein/ml of incubation medium in a final volume of 0.32 ml. The values are the means $\pm$ S.E.M. of the number of experiments from separate preparations listed under 'n'. Abbreviation: ND, not determined.

Substrate	n	O ₂ consumption (nmol of O ₂ /min per mg of protein)			
		State 4	State 3	State 3/State 4	ADP/O
Endogenous		≤ 1	≤ 1	ND	ND
α -GP (+ EGTA)	4	31 ± 3	41 ± 5	1.3 ± 0.1	ND
α -GP (– EGTA)	5	71 ± 8	153 ± 8	2.2 ± 0.2	1.2 ± 0.1
Succinate	12	39 ± 2	183 ± 12	4.7 ± 0.2	2.0 ± 0.1
2-OG	4	13 ± 1	99 ± 8	7.7 ± 0.7	2.9 ± 0.1
Glutamate + malate	8	20 ± 2	118 ± 6	6.4 ± 0.4	2.8 ± 0.1
Glutamate	5	11 ± 2	31 ± 6	2.9 ± 0.4	ND
Pyruvate	6	11 ± 1	15 ± 1	1.5 ± 0.2	ND
Pyruvate + malate	5	18 ± 3	86 ± 8	5.0 ± 0.4	2.2 ± 0.1
Malate (0.5 mM)	3	10 ± 1	15 ± 1	1.5 ± 0.5	ND

pendence varied with the Ca^{2+} level (Figure 2A), with half-maximal values of 2.5 mM at 100 nM free Ca^{2+} and 1.0 mM at 1 μM free Ca^{2+} (Figure 2B). The maximal calculated flux was about 20 nmol/min per mg of cell protein and was independent of the Ca^{2+} concentration. At 1 mM $\alpha\text{-GP}$, respiration was enhanced by Ca^{2+} (Figure 2C) with a half-maximal effect at around 300 nM free Ca^{2+} (Figure 2D). This suggests that physiological changes of Ca^{2+} would exert important modulatory effects on flux through $\alpha\text{-GP}$ dehydrogenase. These results confirm earlier reports of Ca^{2+} -sensitive respiration from $\alpha\text{-GP}$ in β -cell mitochondria [9,12,13] and demonstrate the concentration-dependence for both $\alpha\text{-GP}$ and Ca^{2+} . The calculated apparent maximal flux varied somewhat among cell preparations; this is responsible for the different values in the different panels.

It is well established that Ca^{2+} can also activate PDH [5,6]. We sought to compare the ability of Ca^{2+} to stimulate respiration from pyruvate, the substrate of the intramitochondrial PDH, with that from $\alpha\text{-GP}$. Permeabilization was performed in the presence of EGTA to prevent accumulation of Ca^{2+} by the mitochondria, and Ca^{2+} was raised to approx. 1 μM after establishment of the State 3 rate of respiration in the presence of ADP and the substrate alone. Table 2 demonstrates that, under the experimental conditions used, Ca^{2+} had no significant effect on O_2 consumption in response to pyruvate, whereas a marked increase in respiration from $\alpha\text{-GP}$ was observed (see Figure 2). Since high concentrations of pyruvate may activate PDH or mask activation by other modulators [22], a concentration of pyruvate (0.1 mM) was chosen to stimulate respiration significantly but not maximally (Table 2). Assessment of Ca^{2+} effects on respiration from lower (20, 50 μM) and higher (1, 5 mM) concentrations of pyruvate also showed no effect of Ca^{2+} (results not shown).

Since mitochondrial dehydrogenases are also regulated by the ATP/ADP ratio and mitochondrial redox state [29], we considered the possibility that full activation of PDH had already occurred and prevented any further increment of activity by the increase in Ca^{2+} . To assess the possible influence of these parameters on the Ca^{2+} sensitivity of PDH, we varied the ATP/ADP ratio, by adding either acetoacetate to oxidize or β -hydroxybutyrate to reduce the mitochondrial pyridine nucleotides. As expected, increases in the ATP/ADP ratio decreased respiration from all substrates (results not shown). Addition of β -hydroxybutyrate or acetoacetate rapidly altered the pyridine nucleotide oxidation state (Figure 3A). This indicated that β -hydroxybutyrate dehydrogenase is near equilibrium in the clonal β -cells. Under these conditions of fixed ATP/ADP ratios and relatively reduced or oxidized pyridine nucleotides, respiration from $\alpha\text{-GP}$, but not pyruvate, responded to an increase in Ca^{2+} with an increase in respiration (results not shown). These findings demonstrate that under our experimental conditions an increase in cytosolic Ca^{2+} did not increase the rate of respiration from pyruvate.

It has been shown in several systems that Ca^{2+} activation of intramitochondrial dehydrogenases causes an increase in the intramitochondrial redox state, presumably as a result of increased flux through the enzymes [29,30]. We, therefore, evaluated the effect of Ca^{2+} on pyridine nucleotide fluorescence in permeabilized clonal β -cells oxidizing pyruvate. Figure 3(B) illustrates that addition of pyruvate caused an increase in pyridine nucleotide reduction that increased further upon addition of Ca^{2+} . These results suggest that Ca^{2+} indeed activates PDH, but the data in Figure 3(B) also demonstrate that this activation did not result in a measurable increase in respiration.

To corroborate further the puzzling observation that Ca^{2+} activated PDH without increasing respiration, we measured flux

Figure 3 Effect of acetoacetate (Acoc), β -hydroxybutyrate (βOHB) (A), pyruvate and Ca^{2+} (B) on mitochondrial oxidation state and on O_2 consumption induced by pyruvate (B) in permeabilized β -cells

The results in (A) show the alterations in pyridine nucleotide fluorescence induced by the addition of 5 mM Acoc to oxidize or βOHB to reduce. The results in (B) show pyridine nucleotide fluorescence using pyruvate as substrate and the accompanying changes in respiration. Experiments were performed in the presence of 0.1 mM pyruvate and a fixed ATP/ADP ratio of 25 (ATP + ADP = 4 mM), at a concentration of 80 nM (basal) or 1 μM Ca^{2+} as indicated by the arrow. Traces are representative of experiments that were repeated with three separate preparations.

through PDH using [$1\text{-}^{14}\text{C}$]pyruvate. These experiments showed that Ca^{2+} caused a 50% increase in $^{14}\text{CO}_2$ production and confirmed the activation of PDH by Ca^{2+} (Table 3). Measurement of citrate output into the medium indicated that Ca^{2+} greatly stimulated citrate production and efflux from the mitochondria by 3.4 nmol/30 min per mg of protein or nearly 90%, when pyruvate was the substrate (Table 3). This accounted for more than 20% of the Ca^{2+} -induced increase (15 nmol/30 min per mg of protein) in flux through PDH. These results document a condition where activation of PDH leads to efflux of pyruvate carbon from the citric acid cycle and may partially explain a failure to find an increase in respiration under these conditions (the open system of the permeabilized cell preparation).

Another consideration relates to the contribution of fatty acids which are the primary fuel in unstimulated β -cells. To determine whether PDH activation influenced fatty acid oxidation, cells were cultured overnight in [$1\text{-}^{14}\text{C}$]palmitate to permit labelling of the endogenous lipid pools. When these cells were permeabilized and incubated in the presence of 0.1 mM pyruvate, fatty acids were converted into $^{14}\text{CO}_2$. Addition of 1 μM Ca^{2+} caused a 25% decrease in $^{14}\text{CO}_2$ production. Since total respiration was unchanged, these results suggest that Ca^{2+} induced a shift from fatty acid oxidation to pyruvate oxidation. Thus, the decrease in fatty acid oxidation induced by Ca^{2+} may also partially explain the failure to find an increase in net respiration when pyruvate flux through PDH was stimulated. It should be noted that the effect of Ca^{2+} on basal fatty acid oxidation in the absence of pyruvate was not determined.

Table 3 The effect of Ca^{2+} on flux through PDH and citrate production in permeabilized clonal pancreatic β -cells (HIT)

Experiments were performed in the presence of 0.1 mM pyruvate, a fixed ATP/ADP ratio of 25 (ATP + ADP = 4 mM), and Ca^{2+} concentrations were 80 nM (basal) or 1 μM (Ca^{2+}). $^{14}\text{CO}_2$ production from pyruvate was determined from permeabilized β -cells incubated in the presence of [^{14}C]pyruvate. Citrate efflux into the medium of permeabilized cells was measured by enzymic analysis. $^{14}\text{CO}_2$ production from palmitate was determined from permeabilized β -cells that had incorporated 170 pmol of palmitate into the total lipid pool by incubating overnight with [^{14}C]palmitate. (No palmitate was present during incubations and the cells had been washed free of label prior to incubation). Values represent means $\pm$ S.E.M. of three experiments from separate preparations.

Condition	Pyruvate to CO_2 (nmol/30 min per mg of protein)	Citrate (nmol/30 min per mg of protein)	Palmitate to CO_2 (pmol/30 min per mg of protein)
Basal	33 ± 1.5	3.9 ± 0.2	0.45 ± 0.01
Ca^{2+}	48 ± 1.1	7.3 ± 0.3	0.33 ± 0.04
% Change	+45	+87	-25

DISCUSSION

The substrates for mitochondrial respiration in β -cells

Our data provide the first comparison of the rates of respiration by different fuels in β -cell mitochondria. These mitochondria respond to fuels like most other mammalian mitochondria that have been studied, with the following exception: α -GP is a much better substrate for mitochondrial respiration (Tables 1 and 2) than it is in any other cell type except brown adipose tissue and L1210 cells [10]. Our studies are consistent with major roles for α -GP and pyruvate, the mitochondrial fuels which are produced from glucose. However, we and others have found that endogenous fatty acids are also major fuels for β -cell energy production since their rate of oxidation is very high in the absence of glucose and β -cells maintain a constant rate of respiration for up to 2 h in the absence of exogenous fuels [31–34]. Oxidation of fatty acids is inhibited by glucose, but only by about 25% in normal [33] and clonal β -cells [27]. Since glucose stimulation of β -cells increases respiration by about 50% and inhibits fatty acid oxidation by 25%, it can be deduced that glucose and endogenous fatty acids are each responsible for consumption of about half of the oxygen used by stimulated cells, assuming no contribution of glycogen or amino acids. Indeed, low levels of glycogen have been reported in β -cells [36], whereas triacylglycerol stores are similar to those found in liver [33].

The role of α -GP in pancreatic β -cells

Our findings suggest that the α -GP shuttle, through the high levels of cytosolic and mitochondrial α -GP dehydrogenases [11], may form an important link between the triose phosphates, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (produced during glycolysis in the cytosol), and stimulation of mitochondrial respiration. This role of α -GP and its high potential rate of respiration could serve four purposes: first, to shuttle electrons (reducing equivalents) directly from the cytosol to the mitochondria; secondly, by regenerating NAD^+ in the cytosol to maintain the cytosolic redox state, it permits high rates of glycolysis that might otherwise be diminished by accumulation of NADH, which is a potent product inhibitor of glyceraldehyde-3-phosphate dehydrogenase; thirdly, it conserves carbon in the β -cell by transferring reducing equivalents and carbon to the mitochondria rather than permitting reduction of pyruvate to lactate, which is not a dominant pathway in islets because of the low levels of lactate dehydrogenase activity [37]; and fourthly, the accompanying flavin reduction, when electrons enter the respiratory chain at site two, could provide a rapid signal to the ATP synthase to accelerate ATP production. The latter may be of particular importance in early conversion of ADP into ATP in order to close ATP-sensitive K^+ channels, leading to cell mem-

brane depolarization and influx of Ca^{2+} through voltage-dependent channels, thereby activating both the mitochondrial dehydrogenases and the secretory machinery.

Estimates of the relative contributions of α -GP, pyruvate and fatty acid oxidation to O_2 consumption

An estimate of the effect of glucose on flux through α -GP dehydrogenase can be made based on increases in islet concentrations of Ca^{2+} [23] and α -GP in response to a switch from substimulatory (3 mM) to stimulatory (20 mM) glucose concentrations. Ca^{2+} increases from 100 to 300 nM (3-fold) [23] and α -GP increases from 0.35 to 0.6 mM (1.7-fold) [15]. Since these levels are below their calculated half-maximal values for activation, the response should be approximately proportional to both changes, leading to a 5-fold increase in flux through the mitochondrial α -GP dehydrogenase. This value is in agreement with previous flux estimates using a tracer technique [15]. It should be noted that the measurements of whole islet levels of α -GP may be an underestimate of the actual change since the other cell types contained in the islet presumably do not respond to glucose with an alteration in α -GP. Hence, the estimated 5-fold increase in flux through the mitochondrial α -GP dehydrogenase is probably a minimum value.

The relative contribution of α -GP oxidation to the observed increase in O_2 consumption caused by glucose may, however, be small. Thus, α -GP shuttle activity in intact cells is dependent on the rate of cytosolic NADH production by glycolysis. Rates of α -GP and pyruvate oxidation are likely to be the same since lactate dehydrogenase levels are very low in β -cells [37], with pyruvate consuming much more O_2 than α -GP. Electrons entering the respiratory chain from α -GP at the flavin step consume half a molecule of O_2 to generate two molecules of ATP, whereas electrons entering the respiratory chain from complete pyruvate oxidation consume two and a half molecules of O_2 to generate 14 molecules of ATP. Consequently, α -GP oxidation would consume 17% and pyruvate 83% of the O_2 if glucose were the only fuel and half that amount if fatty acids contributed 50% as suggested above. Indeed, considering the well-documented decrease in fatty acid oxidation in response to glucose [27], it should be noted that both pyruvate and α -GP must be important mitochondrial fuels and contribute to the increase in respiration stimulated by glucose.

The role of Ca^{2+} , redox state and malonyl-CoA in regulating mitochondrial metabolism

The failure to observe increased net respiration from pyruvate in response to increases in extramitochondrial Ca^{2+} does not mean that this treatment failed to activate PDH. It should be noted that the actual change in mitochondrial Ca^{2+} was not determined

in these studies although others have shown that increases in intramitochondrial Ca^{2+} levels occur when extramitochondrial Ca^{2+} levels increased [14]. Consistent with that expectation, we (Figure 3C) and others [10] have shown an increase in mitochondrial pyridine nucleotide fluorescence in response to Ca^{2+} , with pyruvate as substrate, as well as an increase in CO_2 production from [$1\text{-}^{14}\text{C}$]pyruvate (Table 3), suggesting PDH activation. We also showed previously that acetyl-CoA levels do not decrease in the presence of stimulatory glucose despite inhibition of fatty acid oxidation [27,32], a finding consistent with PDH activation. The possibility that a limitation at site I of the respiratory chain could account for the lack of increased respiration in response to Ca^{2+} is ruled out by the demonstration that higher concentrations of pyruvate stimulate higher rates of respiration (Table 2). The possibility that an increase in respiration from pyruvate is compensated by a decrease in fatty acid oxidation must be considered, particularly since glucose has been shown to decrease fatty acid oxidation in intact β -cells [27].

Our demonstration that Ca^{2+} inhibits fatty acid oxidation in permeabilized cells in the presence of pyruvate suggests a novel role for the cation in switching between fatty acid oxidation and pyruvate oxidation. The mechanism for this shift is likely to be reduction of the mitochondrial pyridine and flavin nucleotides, which inhibit fatty acid oxidation [38]. In the intact cell, glucose induced inhibition of fatty acid oxidation is believed to be mediated by malonyl-CoA, formed from citrate [27,32,39]. However, in the open environment of the permeabilized cell malonyl-CoA could not reach an inhibitory concentration because of rapid equilibration into the extracellular medium as well as the dilution of the limited cellular pool of CoA. In light of the current data, consideration must be given to a contribution of redox in control of fatty acid oxidation in the intact glucose-stimulated β -cell. Thus, glucose-induced inhibition of fatty acid oxidation in the intact cell may represent a co-operative effect of malonyl-CoA and increases in mitochondrial redox state.

An additional effect of Ca^{2+} activation of PDH may be related to the so-called 'anaplerotic/malonyl-CoA' pathway in β -cell signalling [40]. The Ca^{2+} activation of PDH could permit a higher steady-state concentration of acetyl-CoA (which is needed to activate pyruvate carboxylase), thus permitting anaplerotic influx of pyruvate carbon into the citric acid cycle as oxaloacetate. The rise in both substrates for citrate synthase should then lead to increased citrate production as we have shown (Table 3). Here we propose that conversion of pyruvate into both oxaloacetate and acetyl-CoA favours citrate production and subsequent efflux from the mitochondria. This pathway is essential for the previously observed accumulation of malonyl-CoA and ensuing generation of cytosolic acyl-CoA-derived metabolic coupling factors [27,32,39]. It should be mentioned that isolated pancreatic islet mitochondria exported large amounts of malate and little or no citrate to the medium [41]. As shown here, it appears that β -cell mitochondria in a more preserved environment (permeabilized cells) are quite capable of exporting citrate.

The role of ADP in regulating mitochondrial metabolism

It is well established that glucose induces an increase in the ATP/ADP ratio (a decrease in ADP) in β -cells [17–19] and this should inhibit rather than stimulate respiration. Indeed, we have documented that respiration in the permeabilized clonal β -cells decreased with increased ATP/ADP ratios. However, it should be noted that reliable determination of cytosolic free ADP levels has not yet been achieved in pancreatic β -cells. The paradox that enhanced O_2 consumption requires a rise in the ADP concentration, whereas closure of ATP-sensitive K^+ channels

requires a decrease in ADP could be resolved by oscillatory behaviour of glycolysis and the energy state, whereby ADP concentrations alternate between high and low values. The observed oscillations in membrane potential [42,43] and O_2 consumption [23] in intact cells are consistent with such alternating control. Our recent demonstration of oscillations in the ATP/ADP ratio and glucose 6-phosphate level in a suspension of β -cells further supports this proposition [19].

Conclusions

The data are consistent with a model in which Ca^{2+} , substrate supply and ADP all play important roles in glucose-induced insulin release and β -cell mitochondrial metabolism. Glucose would initially produce ADP as a consequence of glucose phosphorylation and ultimately increase the concentration of the mitochondrial substrates α -GP and pyruvate, which together stimulate respiration [17]. The subsequent conversion of ADP into ATP and decrease in free ADP would cause closure of the ATP-sensitive K^+ channels [44] and depolarization of the β -cell, leading to a rise in Ca^{2+} due to opening of voltage-gated Ca^{2+} channels. This would occur in a cyclic or oscillatory manner, accounting for the average increase in the ATP/ADP ratio [45,46]. The increase in Ca^{2+} would have at least four effects: (1) it would stimulate the exocytotic apparatus in a manner that is not yet defined; (2) it would activate mitochondrial dehydrogenases including PDH and, indirectly, via acetyl-CoA production, pyruvate carboxylase to promote citrate efflux from the mitochondria and thence malonyl- and cytosolic long-chain acyl-CoA production; (3) it would cause an increase in redox which together with malonyl-CoA would mediate a shift from fatty acid to carbohydrate oxidation; and (4) it would activate the α -GP shuttle.

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REFERENCES

- Welsh, M., Hellerström, C. and Andersson, A. (1982) *Biochim. Biophys. Acta* **721**, 178–184
- Panten, U. and Klein, H. (1982) *Endocrinology* **111**, 1595–1600
- Hutton, J. C. and Malaisse, W. J. (1980) *Diabetologia* **18**, 395–405
- Prentki, M. and Matschinsky, F. M. (1987) *Physiol. Rev.* **67**, 1185–1248
- McCormack, J. G., Halestrap, A. P. and Denton, R. M. (1990) *Physiol. Rev.* **70**, 391–425
- Hansford, R. G. (1985) *Rev. Physiol. Biochem. Pharmacol.* **103**, 1–72
- McCormack, J. G., Longo, E. A. and Corkey, B. E. (1990) *Biochem. J.* **267**, 527–530
- MacDonald, M. J. (1981) *J. Biol. Chem.* **256**, 8287–8290
- MacDonald, M. J. (1982) *Horm. Metab. Res.* **14**, 678–679
- Hansford, R. G. (1991) *J. Bioenerg. Biomembr.* **23**, 823–854
- Meglasson, M. D. and Matschinsky, F. M. (1986) *Diabetes Metab. Rev.* **2**, 163–214
- Rasschaert, J. and Malaisse, W. J. (1991) *Biochem. J.* **278**, 335–340
- Rutter, G. A., Pralong, W.-F. and Wollheim, C. B. (1992) *Biochim. Biophys. Acta* **1175**, 107–113
- Rutter, G. A., Theler, J. M., Murgia, M., Wollheim, C. B., Pozzan, T. and Rizzuto, R. (1993) *J. Biol. Chem.* **268**, 22385–22390
- Sener, A. and Malaisse, W. J. (1992) *J. Biol. Chem.* **267**, 13251–13256
- Ashcroft, S. J. H. and Christie, M. R. (1979) *Biochem. J.* **184**, 697–700
- Civelek, V. N., Deeney, J. T., Kubik, K., Schultz, V., Tornheim, K. and Corkey, B. E. (1996) *Biochem. J.* **315**, 1015–1019
- Meglasson, M. D., Nelson, J., Nelson, D. and Erecinska, M. (1989) *Metab. Clin. Exp.* **38**, 1188–1195
- Nilsson, T., Schultz, V., Berggren, P.-O., Corkey, B. E. and Tornheim, K. (1996) *Biochem. J.* **314**, 91–94
- Deeney, J. T., Tornheim, K., Korchak, H. M., Prentki, M. and Corkey, B. E. (1992) *J. Biol. Chem.* **267**, 19840–19845

- 21 Corkey, B. E., Deeney, J. T., Glennon, M. C., Matschinsky, F. M. and Prentki, M. (1988) *J. Biol. Chem.* **263**, 4247–4253
- 22 Williamson, J. R. (1976) in *Mitochondria: Bioenergetics, Biogenesis and Membrane Structure*, pp. 79–107, Academic Press, Inc, New York
- 23 Longo, E. A., Tornheim, K., Deeney, J. T., Varnum, B. A., Tillotson, D., Prentki, M. and Corkey, B. E. (1991) *J. Biol. Chem.* **266**, 9314–9319
- 24 Prentki, M., Janjic, D. and Wollheim, C. B. (1983) *J. Biol. Chem.* **258**, 7597–7602
- 25 Kikuchi, M., Wollheim, C. B., Cuendet, G. S., Renold, A. E. and Sharp, G. W. (1978) *Endocrinology* **102**, 1339–1349
- 26 Williamson, J. R., Corkey, B. E., Martin-Requero, A., Walajtys-Rode, E. and Coll, K. E. (1986) in *Problems and Potential of Branched Chain Amino Acids in Physiology and Medicine* (Odessey, R., ed.), pp. 135–172, Elsevier, North Holland, Amsterdam
- 27 Corkey, B. E., Glennon, M. C., Chen, K. S., Deeney, J. T., Matschinsky, F. M. and Prentki, M. (1989) *J. Biol. Chem.* **264**, 21608–21612
- 28 Williamson, J. R. and Corkey, B. E. (1979) *Methods Enzymol.* **55**, 200–222
- 29 Hansford, R. G. (1980) *Curr. Top. Bioenerg.* **10**, 217–278
- 30 Hansford, R. G. and Castro, F. (1981) *Biochem. J.* **198**, 525–533
- 31 Corkey, B. E., Tornheim, K., Deeney, J. T., Glennon, M. C., Matschinsky, F. M., Ruderman, N. B. and Prentki, M. (1989) in *Integration of Mitochondrial Function* (Lemasters, J. J., Hockenbrock, C. R., Thurman, R. G. and Westerhoff, H. V., eds.), pp. 543–550, Plenum, New York
- 32 Prentki, M., Vischer, S., Glennon, M. C., Regazzi, R., Deeney, J. T. and Corkey, B. E. (1992) *J. Biol. Chem.* **267**, 5802–5810
- 33 Malaisse, W. J., Best, L., Kawazu, S., Malaisse-Lagae, F. and Sener, A. (1983) *Arch. Biochem. Biophys.* **224**, 102–110
- 34 Malaisse, W. J., Malaisse-Lagae, F., Sener, A. and Hellerström, C. (1985) *Biochem. J.* **227**, 995–1002
- 35 Reference deleted.
- 36 Malaisse, W. J., Sener, A., Koser, M., Ravazzola, M. and Malaisse-Lagae, F. (1977) *Biochem. J.* **164**, 447–454
- 37 Sekine, N., Cirulli, V., Regazzi, R., Brown, L. J., Gine, E., Tamarit-Rodriguez, J., Girotti, M., Marie, S., MacDonald, M. J., Wollheim, C. B. and Rutter, G. A. (1994) *J. Biol. Chem.* **269**, 4895–4902
- 38 Williamson, J. R., Scholz, R. and Browning, E. T. (1969) *J. Biol. Chem.* **244**, 4617–4627
- 39 Prentki, M. and Corkey, B. E. (1996) *Diabetes* **45**, 273–283
- 40 Brun, T., Roche, E., Assimacopoulos-Jeannet, F., Corkey, B. E., Kim, K.-H. and Prentki, M. (1996) *Diabetes* **45**, 190–198
- 41 MacDonald, M. J. (1995) *J. Biol. Chem.* **270**, 20051–20056
- 42 Cook, D. L. (1983) *Metab. Clin. Exp.* **32**, 681–685
- 43 Henquin, J. C., Meissner, H. P. and Schmeer, W. (1982) *Pflugers Arch.* **393**, 322–327
- 44 Hopkins, W. F., Fotherazi, S., Peter-Riesch, B., Corkey, B. E. and Cook, D. L. (1992) *J. Membr. Biol.* **129**, 287–295
- 45 Andrés, V., Schultz, V. and Tornheim, K. (1990) *J. Biol. Chem.* **265**, 21441–21447
- 46 Tornheim, K., Andrés, V. and Schultz, V. (1991) *J. Biol. Chem.* **266**, 15675–15678