Glycerol 3-phosphate-induced ATP production in intact mitochondria from pancreatic B-cells

Lars-Åke IDAHL* and Nicolas LEMBERT

Department of Histology and Cell Biology, Umeå University, S-90187 Umeå, Sweden

A bioluminescent method is presented that allows monitoring of ATP production from mitochondria corresponding to one islet of Langerhans per sample. In mitochondria from *ob/ob* mice Ca^{2+} stimulates the ATP production in the presence of L-glycerol 3-phosphate (GP) by reducing the K_m for GP by one order of magnitude to about 3 mM. Maximal ATP production in the presence of Ca^{2+} (200 nM) is obtained at 10 mM GP. The free calcium concentration required to reach half-maximal stimulation ($K_{0.5 \text{ Ca}^{2+}}$) depends on the GP concentration, thus half-maximal effects are observed at about 80 nM at low GP (1 mM) and 10 nM at high GP (10 mM). Sodium can replace Ca^{2+} as a

stimulator of GP-induced ATP production. It activates ADP phosphorylation by B-cell mitochondria in a sigmoidal concentration-dependent manner in the absence of Ca^{2+} (Hill coefficient 2.3 ± 0.2) but does not change $K_{0.5 \text{ Ca}^{2+}}$ nor the maximal mitochondrial activity. Ca^{2+} concentrations higher than 300 nM are inhibitory at all tested substrate concentrations. Mitochondria from *ob/ob* mice showed no functional defect when compared with normal controls. It is concluded that activation of the glycerol phosphate shuttle may not be the main coupling site for glucose-induced insulin release at maximal cytoplasmic Ca^{2+} levels.

INTRODUCTION

The pancreatic B-cell serves as a glucostat in the body by balancing the blood-glucose level with insulin release. In non-insulin-dependent diabetes mellitus (NIDDM or type-II diabetes) a defective stimulus–secretion coupling causes uncontrolled glucose changes. A crucial point in the understanding of that malfunction is the mechanism of metabolic activation in the B-cell.

Several observations indicate that insulin release is tightly connected to the metabolic degradation of the nutrient stimulus. On glucose stimulation, the healthy B-cell increases the ATP content [1]. The closing of ATP-dependent K^+ channels initiates Ca^{2+} influx [2], which eventually triggers insulin release. Thus mitochondrially produced ATP has been proposed to be a main coupling factor between glycolytic intermediates and insulin release [3].

Pancreatic B-cells are equipped with an exceptionally high content of mitochondrial L-glycerol-3-phosphate dehydrogenase [(GPDH)m] [4]. It was therefore suggested that glucose-induced stimulation of the B-cells may act on the mitochondria through the glycerol phosphate shuttle. The enzyme is located at the outside of the inner mitochondrial membrane and possesses a calmodulin-like Ca^{2+} -binding site [5]. The activation of (GPDH)m by Ca^{2+} [6] led to speculations about the role of Ca^{2+} in the early stages of stimulus–secretion coupling. This question has been re-examined recently [7,8] and a close relationship between cytoplasmic Ca^{2+} concentrations and the mitochondrial activity was suggested. Thus it was proposed that ATP production via the glycerol phosphate shuttle would be stimulated by Ca^{2+} in a feed-forward mechanism.

The present study aims at elucidating the role of Ca^{2+} in the stimulation of intact B-cell mitochondria. However, L-glycerol

3-phosphate (GP)-stimulated mitochondrial ATP production depends not only on (GPDH)m activity but is rather an integrated functional signal. Ca^{2+} is also transported into the matrix where it can regulate, for example, the respiratory chain activity [9]. Thus matrix Ca^{2+} may influence mitochondrial ATP production in response to GP even if (GPDH)m faces the outside of the inner mitochondrial membrane and primarily responds to cytosolic Ca^{2+} . Sodium promotes Ca^{2+} efflux from the mitochondria, thus lowering the matrix Ca^{2+} concentration. In order to prevent Ca^{2+} accumulation in the matrix, sodium was included in the assay.

Mitochondrial ATP production on stimulation with GP was monitored using the luciferin–luciferase system. Due to the limited amount of mitochondria available from pancreatic B-cells, the original method [10] designed for isolation and monitoring of ATP production from intact muscle mitochondria had to be refined. In the present study mitochondria corresponding to a single *ob/ob*-mouse islet of Langerhans were used in each determination, but the sensitivity of the method can easily be increased.

MATERIALS AND METHODS

Chemicals

Microbial collagenase P (EC 3.4.24.3), firefly luciferase (EC 1.13.12.7), D-(–)-luciferin, ADP (potassium salt), ATP (potassium salt), GP (tricyclohexylammonium salt) and electrophoretically homogeneous, lyophilized BSA were purchased from Boehringer (Mannheim, Germany) whereas BSA (fraction V) was from Miles Laboratories (Stoke Poges, U.K.). Non-esterified Fura-2 was from Molecular Probe Inc. (Eugene, OR, U.S.A.). Antimycin A was from Sigma (St. Louis, MO, U.S.A.). Hepes was obtained from Calbiochem (La Jolla, CA, U.S.A.). NaCl, KCl, KH_2PO_4 , MgSO_4 and KOH (Suprapur) were from Merck

Abbreviations used: GP, L-glycerol 3-phosphate; GPDH, L-glycerol-3-phosphate dehydrogenase; (GPDH)m, mitochondrial GPDH; $K_{0.5 \text{ Ca}^{2+}}$, free Ca^{2+} concentration required for half-maximal response; KRH, Krebs–Ringer buffer; $[\text{Ca}^{2+}]_i$, free Ca^{2+} concentration; $K_{m \text{ GP}}$, K_m for GP.

* To whom correspondence should be addressed.

(Darmstadt, Germany). MgCl_2 and CaCl_2 (1 M stock solution) were from BDH Chemicals (Poole, Dorset, U.K.). Quartz bidistilled water was used throughout.

Solutions

Krebs–Ringer buffer (KRH) consisted of (mM): 130, NaCl; 4.7, KCl; 1.2, KH_2PO_4 ; 1.2, $\text{MgSO}_4 \cdot 6\text{H}_2\text{O}$ and 20, Hepes. The pH was adjusted to 7.40 with NaOH.

Medium A consisted of (mM): 50, Hepes; 100, KCl; 1.8, ATP; 1, EGTA; 2, MgCl_2 adjusted to pH 7.10 at 37 °C with KOH.

Medium B consisted of (mM): 35, KH_2PO_4 ; 1, EGTA; 2, MgCl_2 ; 30, KCl and 0.5 (mg/ml), albumin (electrophoretically homogeneous) adjusted to pH 7.10 at 37 °C with KOH. In experiments performed at a final sodium concentration of 80 mM, 20 mM KH_2PO_4 was replaced by NaH_2PO_4 and 10 mM KCl by NaCl. To obtain a final sodium concentration of 100 mM, 20 mM KH_2PO_4 was replaced by NaH_2PO_4 and KCl was replaced by NaCl.

GP (0.3–30 mM final concentration) and ADP (6, 20 or 60 μM final concentration) were dissolved in water and if necessary the pH was adjusted to 7.10 with HCl.

The ATP-monitoring reagent was made from medium B by adding 0.04 mg/ml luciferase, 85 μM luciferin and 20 μM antimycin A.

Isolation of pancreatic islets

Adult female *ob/ob* mice from the Umeå colony or their lean litter mates were starved overnight, subjected to light ether anaesthesia and decapitated. After cutting the excised pancreas into pieces (1–2 mm), islets were isolated by digestion at 37 °C in KRH buffer containing 3 mM glucose, 1% (w/v) albumin (fraction V) and 1.25 mg/ml collagenase. The collagenase isolation [11] was stopped after 16 min of continuous shaking by washing the digest several times with ice-cold KRH containing 1% albumin (fraction V). Medium A was used as the final washing buffer. Medium-sized islets devoid of exocrine parenchyma were transferred manually to fresh ice-cold medium A.

Preparation of mitochondria

About 200 isolated islets from *ob/ob* mice or 200–250 islets from lean mice were suspended in 200 μl of medium A and gently homogenized using a Polytron Pt 1200 (Kinematica AG, Basel, Switzerland). The homogenate was centrifuged for 30 s. The supernatant containing the mitochondria was centrifuged for another 2.5 min. The resulting pellet was dissolved in 200 μl of medium A and centrifuged again for 2.5 min. After removing the supernatant, the pellet was dissolved in 200 μl (lean mice) or 1 ml (*ob/ob* mice) of medium B to obtain a suitable suspension of mitochondria. A 152 Microfuge (Beckman Instruments Inc., Palo Alto, CA, U.S.A.) rotating at 12560 rev./min with $r_{\text{av.}}$ of 4.5 cm (7950 g) was used throughout. The entire preparation was performed at 4 °C.

Calculation of free Ca^{2+} concentration $[\text{Ca}^{2+}]$, in the incubation medium

Binding constants of EGTA towards Ca^{2+} and Mg^{2+} were obtained from Sillén and Martell [12] and adjusted to 37 °C according to Van't Hoff's equation

$$\log \frac{K_2}{K_1} = -\frac{\Delta H^\circ}{2.303 R} \left(\frac{1}{T_2} - \frac{1}{T_1} \right) \quad (1)$$

Table 1 Influence of temperature and pH on the binding constants of Mg^{2+} and Ca^{2+} towards EGTA

Values for $\log[K(\text{M}^{-1})]$ and ΔH° ($\text{kJ} \cdot \text{mol}^{-1}$) determined at 20 °C were obtained from Sillén and Martell [12]. These values were used to calculate the binding constants at 37 °C and the apparent binding constants (K_{app}) at 20 °C or 37 °C at pH 7.10.

Cation	ΔH°	$\log K$		$\log K_{\text{app}}$	
		20 °C	37 °C	20 °C	37 °C
H^+	−5.84	9.46	9.40		
H^+	−5.76	8.78	8.72		
Ca^{2+}	−8.38	10.97	10.89	8.33	6.96
Mg^{2+}	5.18	5.20	5.25	1.42	1.32

with K_1 (M^{-1}) being the binding constant and ΔH° the enthalpy ($\text{J} \cdot \text{mol}^{-1}$) determined at T_1 (293 K). R is the gas constant ($8.31 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$).

The pH dependency of binding constants was calculated as described previously [13], where the apparent binding constant between EGTA and ligand ($K_{\text{MeI}'}$) can be calculated according to eqn. (2).

$$\frac{K_{\text{MeI}}}{K_{\text{MeI}'}} = 1 + [\text{H}^+]K_1 + [\text{H}^+]^2K_1K_2 \quad (2)$$

K_1 and K_2 are the proton-binding constants of EGTA and K_{MeI} is the metal-binding constant of EGTA at 37 °C. Table 1 summarizes the binding constants obtained from [12], the corrected values for 37 °C and the apparent binding constants of Mg^{2+} and Ca^{2+} to EGTA at pH 7.10 and 20 °C or 37 °C. These constants were used to calculate $[\text{Ca}^{2+}]$, according to the procedure outlined elsewhere [13]. Solutions of different $[\text{Ca}^{2+}]$, were prepared in Medium B and compared with an independent Ca^{2+} determination using the Fura-2 method.

Determination of $[\text{Ca}^{2+}]$, in the incubation medium

Fura-2 was dissolved in DMSO (1 mg/ml). A Fura-2-containing solution B (90 μl solution B plus 10 μl Fura-2 solution) was pipetted on to a coverglass which was then mounted as the bottom of an open perfusion chamber. The chamber was placed on the object stage of an inverted microscope (Zeiss IM) with an XBO 75W/2 xenon arc lamp and equipped for dual wavelength epifluorescence microfluorometry. Measurements were performed at room temperature (20 °C) with the heating system turned off or at 37 °C by warming the object stage.

At each observation, $[\text{Ca}^{2+}]$, was calculated from the ratio of emitted light at 510 nm measured with a photomultiplier when the coverglass was illuminated with 340 nm or 380 nm excitation light. Filters, filter changer and signal processor were from Zeiss (MSP 21). Measurements were made at the two wavelengths every 5 s using flashes of 125 ms at each wavelength with 200 ms between the flashes. The Fura-2 signal was calibrated as described earlier [14,15] using a K_D of 231 nM [16].

Assay procedure

Medium B (915 μl) was mixed in an Ellermann tube with 10 μl of ADP solution, 10 μl of CaCl_2 solution and 35 μl of GP solution. An aliquot (25 μl) of a 2 M salt solution consisting of NaCl and/or KCl was added to obtain the desired final sodium concentration. The mixture was allowed to equilibrate at 37 °C. The incubation was then started by adding 5 μl of cold mito-

chondrial suspension. The incubation was stopped by adding 25 μ l of ATP-monitoring reagent. After cooling to room temperature the Ellermann tube was transferred to a Packard Tri-Carb liquid-scintillation spectrometer (Model 3310) operated out of coincidence. The bioluminescence was integrated over a period of 6 s.

Data processing and statistics

In each experiment duplicate samples were carried through the entire assay procedure. From the differences between duplicate samples the random error (S.D.) of a single determination was calculated to be 4 % of the duplicate mean throughout the range of ATP concentrations measured in the present study. The mean value of duplicates was entered as one observation when calculating the means $\pm$ S.E.M. from a number of separate experiments. Each experiment comprised a new islet preparation and fresh solutions. Determination of parameter values $\pm$ S.E.M. of the Hill equation was carried out with the SigmaPlot 4.1 program (Jandel Scientific G.m.b.H., Erkrath, Germany).

RESULTS

To test whether the mitochondrial activity remained stable during the incubation experiments, the total ATP production was measured after different incubation times (Figure 1). Since ADP serves as a substrate for ATP production by adenylate kinase, several concentrations of ADP were tested. At 6, 20 or 60 μ M ADP, the ATP production rate in the presence of GP was constant for at least 30 min. In the following experiments the incubation time was kept at 10 min. Light production from blanks without GP, but containing mitochondria and 6 μ M

Figure 1 ATP production from B-cell mitochondria during incubation at 37 °C

Mitochondria from *ob/ob* mice were incubated for different periods of time with 10 mM GP (○) in the absence of Ca^{2+} and presence of 50 mM Na^+ . The values for the ATP production were normalized with the light intensity obtained after 30 min of incubation. The ATP production from the blank devoid of substrate (■) and the corresponding blank containing 10 mM GP and antimycin A (□) are shown. Solid lines were calculated by linear regression. The symbols represent mean values of duplicate samples.

Figure 2 Correlation between calculated and measured free Ca^{2+} concentrations

Free Ca^{2+} concentrations were calculated using the binding constants given in Table 1 and corrected for a 10 % dilution with the Fura-2 solution. Linear regression (solid line) for Ca^{2+} determinations at 20 °C yields a correlation coefficient of 0.99 and a regression coefficient of 1.13. Filled circles denote Ca^{2+} determinations performed at 37 °C.

ADP, was constant, whereas higher concentrations of ADP resulted in an increasing signal (results not shown). Thus 6 μ M ADP was used in the following experiments. Constant light production was also obtained in the presence of GP and antimycin A, excluding extramitochondrial ATP production from GP.

Since the observed Ca^{2+} effects are critically dependent on the accuracy of the calculated $[\text{Ca}^{2+}]_i$, the validity of these calculations was checked by measuring the free Ca^{2+} concentration in the incubation medium with the Fura-2 technique. As shown in Figure 2, calculated and measured $[\text{Ca}^{2+}]_i$ values are in good agreement, except that Ca^{2+} concentrations below 5 nM were underestimated by the Fura-2 method.

The ATP production from intact B-cell mitochondria stimulated with GP is shown in Figure 3. GP was a poor substrate in the absence of Ca^{2+} . Thus about 5 mM GP was needed to induce detectable ATP production. At 30 mM GP 40 % of V_{max} was obtained but the experimental design prevented the use of GP concentrations high enough to observe a maximal response. Free Ca^{2+} at concentrations of 20 or 200 nM activated GP-induced ATP production, affecting both V_{max} and K_m . Thus at 20 nM Ca^{2+} the apparent K_m for GP ($K_{m\text{GP}}$) was reduced to 3 mM and the apparent maximal activity (80 % of V_{max}) was obtained at 10 mM. An increase to 200 nM Ca^{2+} had no further effect on the $K_{m\text{GP}}$ but increased the activity which was maximal at 10 mM GP.

At high GP concentrations Ca^{2+} inhibited ATP production in a concentration-dependent manner. Increasing the Ca^{2+} concentration from 20 to 200 nM reduced mitochondrial ATP production at 30 mM GP from 80 % to 50 % of the respective V_{max} .

The present study was performed with Umeå *ob/ob* mice. Because the islets of *ob* homozygotes have an unusually high proportion of B-cells [17] they have been extensively used for studies on the pancreatic B-cell function, so far without the

Figure 3 Stimulation by Ca^{2+} of GP-induced mitochondrial ATP production

B-cell mitochondria from *ob/ob* mice were stimulated with various concentrations of GP in the absence (open circles) or presence of 20 nM (filled circles) or 200 nM (open triangles) free Ca^{2+} . The broken line denotes ATP production from B-cell mitochondria of lean control mice in the presence of 200 nM free Ca^{2+} . The experiments were performed in the absence of Na^+ . All values were corrected using internal ATP standards, since high concentrations of GP stimulated the luciferin/luciferase reaction *per se*. The ATP production of *ob/ob* mice and lean control mice was normalized relative to the values obtained at 10 mM GP and 20 nM free Ca^{2+} . Mean values $\pm$ S.E.M. of three experiments.

detection of any basic abnormality. Insulin secretion [18], as well as glucose oxidation rates [19], are normal as compared with lean controls. To verify the suitability of the animal model for the present study some comparative experiments were performed with normal controls. Since pancreatic islets of normal controls are smaller than those from *ob/ob* mice, experiments were performed with mitochondria from five islets per sample. In the presence of 200 nM Ca^{2+} the concentration dependency of GP-induced ATP production from normal mice was rather similar to that from *ob/ob* mice (Figure 3).

Sodium stimulated GP-induced ATP production in the absence of Ca^{2+} (Figure 4). The sigmoidal concentration dependency can be analysed by non-linear curve fitting to the Hill equation. Estimated parameter values for the Hill equation were 0.04 ± 0.01 for the blank and 1.41 ± 0.11 for the $V_{\max}$. Calculations of the K_m for Na^+ and the Hill coefficient yielded the values 71.1 ± 5.2 mM and 2.32 ± 0.15 respectively.

The synergistic effect of Na^+ and Ca^{2+} on the ATP production by GP from B-cell mitochondria is shown in Figure 5(a) for mitochondria from *ob/ob* mice. In the absence of sodium, Ca^{2+} stimulated ATP production both at 1 and 10 mM GP. $K_{0.5 \text{ Ca}^{2+}}$ decreased from 80 nM at 1 mM GP to 10 nM at 10 mM GP. The maximal activation at 1 mM GP reached 30% of that in the presence of 10 mM GP. Maximal activity was obtained at 300 nM Ca^{2+} at low GP concentrations, whereas maximal activity at high GP concentrations was obtained at 50 nM Ca^{2+} . Stimulation by 500 nM Ca^{2+} yielded submaximal ATP production.

Sodium increased the ATP production rate without changing $K_{0.5 \text{ Ca}^{2+}}$. This effect is clearly demonstrated at low GP concen-

Figure 4 Stimulation by Na^+ of GP-induced mitochondrial ATP production

Mitochondria from pancreatic B-cells of *ob/ob* mice were stimulated with 5 mM GP at various Na^+ concentrations. The experiments were performed in the absence of free Ca^{2+} . All values were corrected using internal ATP standards. The ATP production was normalized relative to the values obtained at 100 mM Na^+ . The solid line denotes the least-square fit of the Hill equation. Mean values $\pm$ S.E.M. of three experiments.

trations where the overall activity is lower. At 1 mM GP addition of Na^+ increased the maximal activity to 50% of $V_{\max}$.

Similar additive effects of Ca^{2+} and Na^+ were observed in mitochondria from normal controls (Figure 5b). In the presence of 10 mM GP half-maximal activity was obtained in the absence of free Ca^{2+} ($54 \pm 3\%$ of $V_{\max}$; $n = 3$). Maximal activity was reached at 50 nM Ca^{2+} in the absence of Na^+ . $K_{0.5 \text{ Ca}^{2+}}$ increased to about 50 nM at low substrate concentrations. Maximal activity at low substrate concentration was obtained in the presence of 100–200 nM Ca^{2+} . At 0.3 mM and 1.0 mM GP, 20% and 60% respectively of the maximal activity was observed. Na^+ (20 mM) increased the maximal activity at low substrate concentrations by 25 to 35%.

Mitochondria from *ob/ob* mice, when incubated with 10 mM GP in the absence of Ca^{2+} and Na^+ , displayed less than 15% of maximal ATP production (Figure 3). In contrast, mitochondria from islets of lean mice produced about 50% of maximal ATP under the same conditions (see above). This reflects a somewhat higher sensitivity of the latter mitochondria to stimulation with GP, which is evident from the results presented in Figures 5(a) and 5(b).

DISCUSSION

One problem in physiological studies of intact mitochondria from pancreatic B-cells is the restricted tissue availability. The detection limit for ATP in bioluminometric methods is in the femtomolar range. Since the ATP produced after 10 min of incubation is around 0.1 nmol, as judged from internal ATP standards, the sensitivity may be increased further by increasing the concentration of luciferase or luciferin. Care must be taken, however, to keep the linear dose-response relation of the signal [20]. Provided that proper measures are taken the amount of mitochondria per sample may be substantially reduced.

Mitochondrial destruction due to permeability transition [21] is avoided in the present assay procedure since ice-cold mito-

Figure 5 Mitochondrial GP-induced ATP production in the presence of Ca²⁺ and Na⁺

(a) Mitochondria from pancreatic B-cells of *ob/ob* mice were stimulated with 1 mM GP (squares) or 10 mM GP (circles) at various Ca²⁺ concentrations in the absence (open symbols) or presence of 20 mM Na⁺ (filled symbols). All values were corrected using internal ATP standards. Experiments at each substrate concentration were performed in parallel. The ATP production at 10 mM GP was normalized relative to the values obtained at 50 nM free Ca²⁺. At 1 mM GP the ATP production was normalized relative to the activity observed at 10 mM GP and 10 nM free Ca²⁺. Mean values $\pm$ S.E.M. of three experiments. (b) The corresponding experiments for mitochondria from normal controls. Squares and circles denote stimulation with 1 mM and 10 mM GP respectively. Triangles denote measurements performed at 0.3 mM GP. All values were normalized relative to the activity measured at 10 mM GP in the presence of 50 nM free Ca²⁺. Mean values $\pm$ S.E.M. are given for three experiments performed without sodium (open symbols). Filled symbols represent mean values of two experiments performed with 20 mM sodium in the incubation medium.

chondria are transferred directly into the prewarmed incubation medium. The linear ATP production rate demonstrates an immediate adaptation of the mitochondria making a pre-incubation period unnecessary.

The detection of mitochondrial ATP production offers some advantages as compared with colorimetric [6,8] or radioisotopic [7] enzyme assays. It represents an integrated functional response

and reveals physiological limitations of the organelle that can not be observed otherwise.

In contrast to previous studies on mitochondrial GPDH, the results presented here demonstrate a low GP affinity of B-cell mitochondria. The observed K_{mGP} in the absence of Ca²⁺ is above 30 mM, i.e. almost 10 times higher than observed in previous determinations of GPDH(m) kinetics [6,8]. Addition of Ca²⁺ decreases the K_{mGP} to 3 mM for mitochondria compared with 0.5 mM GP in studies on GPDH(m) [8].

Maximal Ca²⁺ stimulation of ATP production from intact mitochondria is observed at 200–300 nM. This is half the concentration (750 nM) determined for maximal activation of islet GPDH(m) by Rutter et al. [8] and more than 30 times lower than the corresponding concentration (10 μ M) found by MacDonald [6]. Moreover, free Ca²⁺ concentrations above 300 nM inhibit mitochondrial ATP production. This observation is corroborated by experiments with mitochondria isolated from lean mice which display a similar pattern for GP-induced ATP production as found in B-cell mitochondria from *ob/ob* mice. The increased activity at low GP concentrations may reflect the fact that lean mice preparations contain a significantly higher amount of non B-cells.

Sodium activates mitochondrial ATP production in a dose-dependent manner. Values for the total Na⁺ concentration in the B-cells range from 60 mM, measured with integrating flame photometry [22], to 95 mM, when determined with isotope uptake measurements [23]. Estimates of the free Na⁺ concentration range from 14 mM [22] to 30 mM [24] as determined from fluorescent probe spectroscopy and electrophysiological measurements respectively, indicating that most of the Na⁺ is bound in the B-cell. Stimulation with glucose reduces the total Na⁺ concentration by 25% with a maximal effect already at 5 mM glucose [22]. Thus concentrations of free Na⁺ in this range may represent a physiological activator of mitochondrial ATP production and the effects of Ca²⁺ should be studied in the presence of Na⁺. Since EGTA has free access to the inner mitochondrial membrane [25] an increase of the extramitochondrial Ca²⁺ concentration due to ion transport over the 2Na⁺/Ca²⁺ antiporter is unlikely.

A Hill coefficient near 2 indicates the participation of more than one Na⁺ in the stimulation of GP-induced ATP production. Since the effects of Ca²⁺ and Na⁺ are synergistic both ions may act on the same allosteric binding site.

The high apparent $K_{0.5Ca^{2+}}$ at physiological GP concentrations (0.1–0.2 mM [26]) is generally used as an argument for a Ca²⁺-activated GP shuttle, since it predicts stimulation over the whole range of cytoplasmic calcium changes (50–600 nM [27]). Here we demonstrate that the stimulatory Ca²⁺ range appears to be more limited for intact B-cell mitochondria. The observed inhibition of mitochondrial ATP production at 500 nM free Ca²⁺ appears to set an upper limit for Ca²⁺-dependent stimulation at 200–300 nM. This inhibition is probably not due to a harmful Ca²⁺ accumulation in the matrix as observed in rat liver mitochondria [28]. Control experiments showed that the ATP production rates were linear with time at all tested Ca²⁺ concentrations (results not shown), indicating that the observed inhibition represents a true reduction in ATP production capacity.

The relatively limited coupling of GP-induced mitochondrial ATP production to changes in cytoplasmic Ca²⁺ may stress the importance of the NAD⁺-regenerating function of the GP shuttle. In the glucose-stimulated B-cell there is a diminished content of NAD⁺ which may limit glycolysis at the glyceraldehyde-3-phosphate dehydrogenase level and restrict the supply of pyruvate for mitochondrial oxidation [29]. For Ca²⁺ to fulfil the alleged stimulatory effect through increased ATP

availability an efficient coupling of the free Ca^{2+} level to the oxidation of other mitochondrial fuels might be necessary. This remains to be tested in future experiments.

This work was supported by grants from the Swedish Medical Research Council (12X-4756), the Swedish Diabetes Association, the Sahlberg Fund and the Medical Faculty at Umeå University. We thank Mrs. Barbro Borgström for excellent technical assistance and Mrs. Ewa Hallin and Dr. Per Lindström for performing the Fura-2 measurements.

REFERENCES

- Hellman, B., Idahl, L.-Å. and Danielsson, Å. (1969) *Diabetes* **18**, 509–516
- Ashcroft, F. M. (1988) *Annu. Rev. Neurosci.* **11**, 97–118
- Malaisse, W. J. (1992) *Int. J. Biochem.* **24**, 693–701
- MacDonald, M. J. (1981) *J. Biol. Chem.* **256**, 8287–8290
- Brown, L. J., MacDonald, M. J., Lehn, D. A. and Moran, S. M. (1994) *J. Biol. Chem.* **269**, 14363–14366
- MacDonald, M. J. (1982) *Horm. Metab. Res.* **14**, 678–679
- 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
- McCormack, J. G., Halestrap, A. P. and Denton, R. M. (1990) *Physiol. Rev.* **70**, 391–425
- Wibom, R., Lundin, A. and Hultman, E. (1990) *Scand. J. Clin. Lab. Invest.* **50**, 143–152
- Lacy, P. E. and Kostianovsky, M. (1967) *Diabetes* **16**, 35–39
- Sillén, L. G. and Martell, A. E. (1971) *Stability Constants of Metal-Ion Complexes*, Special Publications Nos. 17 and 25, The Chemical Society, Burlington House, London
- Pershadsingh, H. A. and McDonald, J. M. (1980) *J. Biol. Chem.* **255**, 4087–4093
- Grapengiesser, E., Gylfe, E. and Hellman, B. (1989) *Arch. Biochem. Biophys.* **268**, 404–407
- Gryniewicz, G., Poenie, M. and Tsien, R. Y. A. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Johansson, H., Larsson, R., Nygren, P., Lindh, E., Rastad, J., Åkerström, G. and Gylfe, E. (1987) *Biosci. Rep.* **7**, 705–712
- Hellman, B. (1965) *Ann. N. Y. Acad. Sci.* **131**, 541–558
- Hellman, B., Idahl, L.-Å., Lernmark, Å., Sehlin, J. and Täljedal, I.-B. (1974) *Arch. Biochem. Biophys.* **162**, 448–457
- Hellman, B., Idahl, L.-Å., Lernmark, Å., Sehlin, J. and Täljedal, I.-B. (1974) *Biochem. J.* **138**, 33–45
- Lambert, N. and Idahl, L.-Å. (1995) *Biochem. J.* **305**, 929–933
- Gunter, T. E. and Pfeiffer, D. R. (1990) *Am. J. Physiol.* **258**, C755–C786
- Ali, L., Grapengiesser, E., Gylfe, E., Hellman, B. and Lund, P. E. (1989) *Biochem. Biophys. Res. Commun.* **164**, 212–218
- Sehlin, J. and Täljedal, I.-B. (1974) *FEBS Lett.* **39**, 209–213
- Meissner, H. P., Henquin, J. C. and Preissler, M. (1978) *FEBS Lett.* **94**, 87–89
- Igbavboa, U. and Pfeiffer, D. R. (1988) *J. Biol. Chem.* **263**, 1405–1412
- Matschinsky, F. M., Gosh, A. K., Meglasson, M. D., Prentki, M., June, V. and Allman, D. V. (1986) *J. Biol. Chem.* **261**, 14057–14061
- Grapengiesser, E., Gylfe, E. and Hellman, B. (1988) *Biochem. Biophys. Res. Commun.* **151**, 1299–1304
- McCormack, J. G. (1985) *Biochem. J.* **231**, 581–595
- Hellman, B., Idahl, L.-Å., Sehlin, J. and Täljedal, I.-B. (1975) *Diabetologia* **11**, 495–500