Matrix alkalinization: a novel mitochondrial signal for sustained pancreatic β -cell activation

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Nutrient secretagogues activate mitochondria of the pancreatic β -cell through the provision of substrate, hyperpolarisation of the inner mitochondrial membrane and mitochondrial calcium rises. We report that mitochondrial matrix pH, a parameter not previously studied in the β -cell, also exerts an important control function in mitochondrial metabolism. During nutrient stimulation matrix pH alkalinises, monitored by the mitochondrial targeted fluorescent pH-sensitive protein mtAlpHi or ³¹P-NMR inorganic phosphate chemical shifts following saturation transfer. Compared with other cell types, the resting mitochondrial pH was surprisingly low, rising from pH 7.25 to 7.7 during nutrient stimulation of rat β -cells. As cytosolic alkalinisation to the nutrient was of much smaller amplitude, the matrix alkalinisation was accompanied by a pronounced increase of the Δ pH across the inner mitochondrial membrane. Furthermore, matrix alkalinisation closely correlates with the cytosolic ATP net increase, which is also associated with elevated ATP synthesis rates in mitochondria. Preventing Δ pH increases in permeabilised cells abrogated substrate-driven ATP synthesis. We propose that the mitochondrial pH and Δ pH are key determinants of mitochondrial energy metabolism and metabolite transport important for cell activation.

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Introduction

The pancreatic β -cell senses a large number of signals, including peptide hormones, neurotransmitters and nutrients, thereby adjusting insulin granule exocytosis to the requirements of the organism to regulate glucose homeo-

stasis. Oxidative metabolism of nutrients causes a plethora of intracellular changes resulting in β -cell activation (Hellman *et al*, 1974; Detimary *et al*, 1996; Maechler and Wollheim, 1999; Wollheim and Maechler, 2002; Cline *et al*, 2004; Ronnebaum *et al*, 2006). Mitochondria sense increased substrate availability and nutrient-derived signals, such as the increase in mitochondrial calcium, required to augment the rate of CO₂ production during nutrient stimulation (Wollheim and Maechler, 2002; Wiederkehr and Wollheim, 2006). The resulting synthesis of ATP and intermediary metabolites act as signals linking nutrient metabolism to insulin granule exocytosis (Detimary *et al*, 1996; Kennedy *et al*, 1999; Ishihara *et al*, 2003). ATP causes closure of the K_{ATP} channels. This in turn results in the depolarisation of the plasma membrane potential, and calcium enters through voltage-gated channels triggering insulin granule exocytosis (Wollheim and Maechler, 2002; Wiederkehr and Wollheim, 2008). Glucose further stimulates insulin secretion by the amplifying pathway (Gembal *et al*, 1992), possibly depending on signaling through metabolic intermediates (Maechler and Wollheim, 1999; Ivarsson *et al*, 2005; Joseph *et al*, 2006; Wiederkehr and Wollheim, 2006). Consistent with this proposal, mitochondrial metabolite transport is required for efficient glucose-dependent insulin secretion (Joseph *et al*, 2006; Ronnebaum *et al*, 2006).

Transport of metabolites across the inner mitochondrial membrane is an active process linked directly or indirectly to the proton electrochemical gradient. According to the Nernst equation, this potential is composed of an electrical and a chemical component responsible for the transport of different sets of metabolites (Simpson and Hager, 1984; Palmieri, 2004). As the electrical and chemical potentials influence the export of ATP, metabolites and ions differentially, they may have different impacts on the export of these from the mitochondria and on their ability to act as signals in metabolism–secretion coupling.

The electrical potential across the inner mitochondrial membrane of the β -cells is augmented during nutrient stimulation (Duchen *et al*, 1993; Maechler and Wollheim, 1999). This hyperpolarisation increases the driving force on the ATP synthase and accelerates electrogenic transport steps such as the export of ATP in exchange for ADP (Das, 2003; Palmieri, 2004).

The proton chemical gradient is proportional to the pH difference between the mitochondrial matrix and the cytosol (Δ pH) and serves as the driving force for the import and export of a large number of metabolites across the inner mitochondrial membrane, also contributing to the driving force on the ATP synthase (Simpson and Hager, 1984; Das, 2003; Palmieri, 2004). Through its effect on metabolite transport, the Δ pH may be important for the amplifying pathway of insulin secretion.

The cytosolic pH (pH_{cyto}) is close to neutral (pH 7.0–7.2) in various mammalian cell types. In the β -cell, pH_{cyto} undergoes a slight alkalinisation from pH 7.0 to 7.1 in response to

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glucose (Shepherd and Henquin, 1995; Stienet *et al*, 2007). In contrast to the pH_{cyto} , the mitochondrial pH (pH_{mito}) has been found to be quite alkaline, ranging from 7.6 to 8.3 in a number of different cells (Llopis *et al*, 1998; Matsuyama *et al*, 2000; Abad *et al*, 2004; Balut *et al*, 2008). The dynamics of pH_{mito} changes with regard to their role in cell activation have not been studied to date.

Results

Metabolically active inorganic phosphate pools in INS-1 cells

^{31}P -NMR provides a non-invasive means of measuring intracellular pH. In particular, the chemical shift of inorganic phosphorus has been shown to be pH dependent (Gadian *et al*, 1979; Petroff *et al*, 1985). Intracellular pH was measured in INS-1 cells based on the chemical shift in the ^{31}P -NMR spectra of intracellular inorganic phosphate (Pi). Pi-pH titration curves were obtained from the Pi and the α -phosphate of ATP chemical shift differences (see Materials and methods). The chemical shifts of α -phosphate of ATP (10.2 p.p.m.) remain constant over the range of pH changes studied here (Gadian *et al*, 1979). ^{31}P -NMR measurements of the intracellular pH in INS-1 cells were obtained at resting glucose concentrations in the perfusion buffer maintained at pH 7.3. Under these conditions, the extracellular Pi peak appears at 2.72 p.p.m., and an intracellular Pi pool appears at 2.39 p.p.m. (Figure 1A). In the standard NMR spectrum, any intracellular Pi pool with a pH between 7.2 and 7.4 co-resonates with the extracellular Pi. The only intracellular Pi pool that is readily observable is the one that appears at 2.39 p.p.m., corresponding to a pH of 6.97, a value in agreement with the expected pH_{cyto} . By performing saturation transfer

experiments, we were able to detect and identify ^{31}P -NMR resonances that correspond to Pi pools that are metabolically active as reactants for the synthesis of ATP from ADP and Pi (Cohen *et al*, 1978; Cline *et al*, 2001). We observed two metabolically active Pi pools, one at 2.37 p.p.m. and the other co-resonating with the extracellular Pi (Figure 1B). On increasing the glucose concentration to 15 mM, we observed an ~ 10 -fold drop in the intracellular Pi signal at 2.37 p.p.m., and the appearance of a downfield shoulder on the extracellular Pi peak (Figure 1C). Interestingly, the saturation transfer experiment revealed a shift of the downfield metabolically active Pi peak by another 47 Hz (Figure 1D). The downfield shift in the resonance suggests that this metabolically active Pi pool senses alkalisation of a specific intracellular compartment. On increasing the glucose concentration from 2 to 15 mM, we observed an average alkalisation from pH 7.45 to 7.73 associated with this Pi pool, whereas the Pi peak at 2.39 p.p.m. was only little affected when elevating the glucose concentration (Table I). The different Pi pools could correspond to different intracellular compartments, raising the question which compartment was undergoing alkalisation during glucose stimulation.

Table I ^{31}P -NMR-measured cytosolic and mitochondrial pH in INS-1 cells

Glucose concentration	Cytosolic	Mitochondrial
Glc 2.5 mM ($n = 5$)	6.95 ± 0.03	7.45 ± 0.13
Glc 15 mM ($n = 5$)	7.01 ± 0.04	$7.73 \pm 0.13^*$

pH was determined from the chemical shift frequency of Pi referenced to the α -phosphate of ATP.

Values are reported as mean $\pm$ s.e.m.

* $P < 0.05$ compared with basal measurement.

Figure 1 ^{31}P -NMR spectra revealing different phosphate pools in INS-1 cells. Representative ^{31}P -NMR spectra of alginate-entrapped INS-1 cells. The cells were perfused in oxygenated KRB buffer containing 2.5 mM glucose (A, B) or 15 mM glucose (C, D). The control spectra of the saturation transfer experiment, with the saturation pulse downfield of Pi at 2.5 mM glucose (A) and 15 mM glucose (C), show resonances corresponding to the extracellular Pi at 2.73 p.p.m. and intracellular Pi at 2.39 p.p.m. The difference between the two spectra of the saturation transfer experiment (saturation pulse at β -ATP or downfield of Pi) reveals metabolically active intracellular Pi pools, with their chemical shifts dependent on the pH of their respective intracellular location. For ease of comparison, difference spectra (B, D) are shown inverted. (In the difference spectra, the β -ATP peaks and Pi peaks have normally negative excursion. The α -ATP peak is not affected by the saturation of the β -ATP resonance and is subtracted out in the difference.)

We speculated that it could be the mitochondrial matrix space because (1) it is a site of active ATP synthesis and (2) the mitochondrial matrix pH is more alkaline than the pH_{cyto} .

Mitochondrial matrix alkalinisation during glucose stimulation of the β -cell

To test this possibility, we set out to measure pH_{mito} using the pH_{mito} probe mtAlpHi. This yellow fluorescent protein-based probe displays a close to linear fluorescence emission increase over a pH range from 7.0 to 8.5 (Abad *et al*, 2004). To express the mtAlpHi not only in cell lines but also in primary β -cells, we re-cloned the transgene into an adenovirus vector (Ad-tetON-mtAlpHi). After infection and induction of the tetON-mtAlpHi virus (see Materials and methods) in primary islet cells and INS-1E cells, mtAlpHi displayed a mitochondrial pattern of localisation (Figure 2A and data not shown) similar to its published distribution in HeLa cells (Abad *et al*, 2004). Insulin immunofluorescence was performed to confirm that mtAlpHi localisation was indeed analysed in β -cells (Figure 2A). To confirm the pH sensitivity of mtAlpHi after adenovirus-mediated overexpression, we used ammonium chloride and FCCP. Ammonium chloride caused a large transient increase of the mtAlpHi fluorescence, consistent with its ability to alkalinise intracellular compartments (Supplementary Figure 1A). Uncoupling by FCCP also dissipates the ΔpH across the inner mitochondrial membrane, causing acidification and therefore a rapid reduction of mtAlpHi fluorescence (Supplementary Figure 1B). The mtAlpHi fluorescence is responsive to pH shifts, in the direction of both acidification and alkalinisation. Remarkably, stimulation of rat islets with 16.7 mM glucose caused an increase of the mtAlpHi fluorescence, suggesting alkalinisation of the matrix space by the nutrient (Figure 2B). Qualitatively similar results were observed after glucose stimulation of INS-1E cells (Supplementary Figure 1C). The onset of the mitochondrial alkalinisation response usually occurred rapidly after a step increase in the glucose concentration. In rat islets, the pH increase appeared more pronounced than in INS-1E cells and took 10–15 min before reaching a new steady state. In some measurements, mtAlpHi fluorescence reached a first maximum to then return to a steady state between basal and maximal fluorescence

(see Figure 3A). To estimate the extent of glucose-mediated alkalinisation, we titrated the mtAlpHi fluorescence as shown in Figure 2B. Using such titration protocols, we find matrix pH to be surprisingly low both in INS-1E ($\text{pH } 7.30 \pm 0.04$; $N=13$; Figure 3B) and in islet cells ($\text{pH } 7.25 \pm 0.05$; $N=6$; Figure 2C). After glucose stimulation, the pH_{mito} increased in INS-1E (Figure 3B; $\text{pH } 7.65 \pm 0.06$; $N=13$) and islet cells (Figure 2C; $\text{pH } 7.70 \pm 0.04$; $N=6$). In the control hepatoma cell line HepG2, matrix pH was alkaline (close to 7.8) independent of the glucose concentration (Figure 3D and

Figure 2 Mitochondrial matrix alkalinisation in response to glucose in rat islets. Dissociated rat islet β -cells (A) or intact rat islets (B, C) were infected with an adenovirus carrying mtAlpHi under the control of the tetON promoter (Ad-tetON-mtAlpHi). Insulin immunofluorescence (A; right panel) was performed to identify β -cells expressing mtAlpHi (A; left panel). (B, C) Infected rat islets were incubated in 2.5 mM glucose and shifted to 16.7 mM glucose as indicated. For titrations of mtAlpHi fluorescence (B; see also Materials and methods), the intra-mitochondrial pH was clamped to pH values as indicated on top of the trace. (C) Average mitochondrial pH responses to glucose in rat islets ($N=7$). (D) Average pH_{cyto} glucose responses in rat islets ($N=9$). The results are expressed as $\pm$ s.e.m. Cytosolic pH (D) was measured using the ratiometric fluorescent probe BCECF. (E, F) Histograms of single-cell analysis of matrix alkalinisation in INS-1E (E) and individual cells in intact rat islets (F). The increase of the pH_{mito} was determined in single INS-1E (55 cells) and rat islets (70 cells from 10 islets). The cells were grouped according to the extent of alkalinisation in 0.1 pH unit increments and expressed as the percentage of total cells analysed.

Figure 3 Mitochondrial pH in INS-1E and HepG2 cells. INS-1E cells (A–C) or HepG2 cells (D, E) expressing mtAlpHi were incubated in 2.5 mM glucose and shifted to 16.7 mM glucose as indicated. Average pH_{mito} changes to glucose $\pm$ s.e.m. in INS-1E cells (B; $N=13$) and HepG2 cells (E; $N=4$) are shown. (C) Average pH_{cyto} changes to glucose $\pm$ s.e.m. in INS-1E cells. Rotenone was used at a concentration of 2.5 μ M.

Table II ^{31}P -NMR-measured ATP synthesis rate parameters in INS-1 cells

Cellular compartment	k_{ATP} (min^{-1})		ATP synthesis rate (mM/min in bioreactor volume)	
	Glc 2.5 mM	Glc 15 mM	Glc 2.5 mM	Glc 15 mM
Cytosolic ($n=4$)	2.4 ± 1.1	$13.7 \pm 3.1^*$	0.58 ± 0.45	1.0 ± 0.37
Mitochondrial ($n=4$)	1.9 ± 1.2	4.6 ± 1.4	0.93 ± 0.61	2.29 ± 0.76

k_{ATP} is the unidirectional rate constant for synthesis of ATP from ADP + Pi. ATP synthesis rate is expressed in terms of mmoles ATP per litre bioreactor volume per minute.

Values are reported as mean $\pm$ s.e.m.

* $P < 0.05$ compared with basal measurement.

E). Addition of the complex I inhibitor rotenone caused a rapid acidification of the matrix pH of HepG2 cells, demonstrating that the matrix pH can be altered in this cell type (Figure 3D). For INS-1E and rat islet, we also analysed the alkalinisation responses of the individual cells (Figure 2E and F). Alkalinisation was observed in the majority of islet cells (Figure 2F; 93%) and INS-1E cells (Figure 2E; 89%). In the rat islets, 63% of the mtAlpHi-positive cells showed alkalinisation responses between 0.2 and 0.6 pH units, with a fraction of the cells (21%) displaying even larger mitochondrial alkalinisation (Figure 2F).

Mitochondrial and cytosolic ATP synthesis rates

The results obtained after expression of mtAlpHi in INS-1 cells are consistent with the Pi shift demonstrating alkalinisation of a specific phosphate pool during glucose stimulation (Figure 1 and Table I). These results would argue that the metabolically active Pi peaks observed at ~ 2.7 p.p.m. and above correspond to the mitochondrial phosphate pool. On the basis of the assignment of cytosolic and mitochondrial Pi pools, we used saturation transfer experiments to determine ATP synthesis rates in the two compartments. Although these experiments provide an absolute value for the unidirectional rate constant for ATP synthesis (k_{ATP}), the actual rates of

cytosolic and mitochondrial ATP synthesis cannot be calculated without knowing the absolute pool sizes. However, as the changes in Pi concentration were calculated from the known concentration of Pi in the extracellular medium, the concentration of Pi in each intracellular compartment can be expressed in terms of the total volume of the bioreactor. This provides a total rate of ATP synthesis from each compartment and allows us to directly compare the total rates of mitochondrial and cytosolic ATP synthesis. For the cytosolic Pi pool at 2.37 p.p.m., we calculated a 5.7-fold increase in the unidirectional rate constant of ATP synthesis from Pi, but because of the significant drop in the NMR-observable intracellular Pi concentration, this translated to a 1.7-fold increase in the ATP synthesis rate (Table II). For the mitochondrial Pi pool at 2.67 p.p.m., we calculated a 2.5-fold increase in both the k_{ATP} and ATP synthesis rates (Table II).

ΔpH increases during glucose stimulation

The observed pH increases in the mitochondrial matrix of the β -cell are quite pronounced, several-fold larger than the published glucose-dependent elevation of the pH_{cyto} (Shepherd and Henquin, 1995; Stiernet *et al*, 2007). To assess pH_{cyto} changes under our experimental conditions, we used ^{31}P -NMR in INS-1 cells (Table I) or the pH-sensitive

fluorescent probe BCECF in INS-1E cells and rat islets (Figures 2D and 3C). Consistent with the published data, we find only a small increase of the pH_{cyto} in both rat islets and INS-1E cells after glucose addition (Table I; Figures 2D and 3C). Direct comparison of pH_{cyto} and pH_{mito} reveals that the ΔpH across the inner mitochondrial membrane is very low at resting glucose concentrations. In rat islet cells, the ΔpH was estimated to be 0.13 pH units. At the stimulatory glucose concentration, a pronounced increase of the ΔpH to 0.51 pH units was measured.

Leucine but not glutamine causes mitochondrial alkalinisation in rat β -cells

To assure that the above-described mitochondrial matrix alkalinisation occurs specifically in the islet β -cells, mtAlpHi was also placed behind the rat insulin promoter (Ad-RIP-mtAlpHi). Although expression was less strong than using the tetON system, proper targeting to mitochondria in INS-1E cells and rat islet cells (Figure 4A) and pH sensitivity of the probe was preserved. Glucose responses in rat islets were indistinguishable from those obtained with Ad-tetON-mtAlpHi (data not shown). Leucine, another insulin secretagogue, induced alkalinisation similar to glucose using Ad-RIP-mtAlpHi (Figure 4B) or Ad-tetON-mtAlpHi (data not shown). Leucine metabolism provides Acetyl-CoA for mitochondrial oxidation, bypassing many reactions required for glucose metabolism. Therefore, the alkalinisation of the matrix is not specifically linked to glucose metabolism but rather to the activation of mitochondrial oxidative metabolism. On the other hand, glutamine, which does not serve as an efficient secretagogue in the β -cell (Sener *et al*, 1981) and does not cause any significant increase in ATP generation (data not shown), did not alkalinise the mitochondrial matrix pH (Figure 4C). The results substantiate the conclusion that the mitochondrial matrix of pancreatic β -cells alkalinises after stimulation with nutrient secretagogues.

Kinetics of matrix pH alkalinisation during metabolism-secretion coupling

To place the observed matrix pH alkalinisation into the context of metabolism-secretion coupling, we compared the average pH response to glucose in rat islets (Figure 5A) with the kinetics of a number of other responses such as insulin secretion (Figure 5B), the mitochondrial calcium rise (Figure 5C) and the augmentation of cytosolic ATP levels (Figure 5D) after glucose stimulation. This comparison reveals that the new steady state of pH_{mito} is reached relatively slowly, lagging for instance behind first-phase insulin secretion (Figure 5A and B). Furthermore, the prominent rise of mitochondrial calcium preceded the alkalinisation response (Figure 5A and C). Given the early mitochondrial calcium rise, we argued that mitochondrial calcium could be an upstream signal required to initiate matrix alkalinisation. To test this possibility, we stimulated rat islets (Figure 6A and B) or INS-1E cells (data not shown) with glucose in the absence of extracellular calcium. Under these conditions, glucose does not raise mitochondrial calcium (data not shown). Despite the absence of the calcium signal, the mitochondrial matrix pH increased rapidly from a pH of 7.22 ± 0.03 ($N=3$) to an average pH of 7.58 ± 0.05 ($N=3$) at high glucose concentration (Figure 6B), a rise which is about 20% smaller than under control conditions. Re-addition of calcium caused a

Figure 4 β -Cell-specific expression of mtAlpHi for the measurement of mitochondrial pH responses to leucine and glutamine. INS-1E cells, dispersed islet cells (A) or intact rat islets (B, C) were infected with an adenovirus expressing mtAlpHi under the control of the rat insulin promoter (Ad-RIP-mtAlpHi). (A) Mitochondrial localisation of mtAlpHi was confirmed in INS-1E and primary rat β -cells. Mitochondrial matrix alkalinisation was induced by leucine (20 mM; B) but not glutamine (10 mM; C).

further small alkalinisation of the pH_{mito} (Figure 6A). The non-nutrient secretagogue tolbutamide, which closes the K_{ATP} channel, thereby raising calcium in the cytosol and mitochondria, did not cause alkalinisation (Figure 6C). Therefore, calcium is neither necessary nor sufficient to cause mitochondrial alkalinisation. We also observed that the glucose-dependent hyperpolarisation of the mitochondrial membrane potential was unchanged in calcium-free medium (Figure 6D). In the dispersed rat islet cells, the membrane potential, monitored ratiometrically with the lipophilic cation JC-1 (Park *et al*, 2008), was elevated by 16% in response to glucose both in the presence and in the absence of extracellular calcium (Figure 6D and E). Re-addition of calcium did not cause depolarisation of the mitochondrial membrane potential. Together with our results on pH_{mito}

changes, this finding shows that hyperpolarisation of the proton electrochemical gradient is only little affected by glucose-dependent calcium signals.

Effect of respiratory chain activity and hyperpolarisation of the mitochondrial membrane on matrix alkalinisation

Next, we tested how mitochondrial respiratory function may relate to matrix alkalinisation. After inhibition of the respiratory chain complexes I by rotenone, a rapid acidification was noticed both in rat islets (Figure 6F) and in INS-1E cells (data not shown). Therefore, respiratory chain-dependent proton pumping is required to maintain the ΔpH . Addition of rotenone at the time of glucose stimulation almost completely suppressed the alkalinisation response to the nutrient in rat islets (Figure 6G). The results show that respiratory chain function is a key driving force for matrix alkalinisation.

Interestingly, the electrical potential and matrix pH changes did not follow the same kinetics during glucose stimulation (Figure 7). While mtAlpHi fluorescence increased quite steadily over a time period of 10 min in INS-1E cells (Figure 7A), the electrical potential as measured with JC-1 initially rose rapidly, followed by a slow second phase (Figure 7B). Half-maximal hyperpolarisation was reached after 88 s. In the case of the pH measurement, the half-maximal response was only reached 270 s after glucose addition. This result argues that net proton extrusion by the respiratory chain from the mitochondrial matrix can be buffered at least during initiation of the response.

Role for matrix pH in mitochondrial ATP generation

We noted a close correlation between alkalinisation and net ATP changes after the glucose stimulus (Figure 5A and D). Our results suggest that mitochondrial alkalinisation could contribute to enhanced energy metabolism. To substantiate this possibility, we performed experiments in permeabilised INS-1E cells, a preparation which allows the manipulation of the pH_{mito} . INS-1E cells were permeabilised with *Staphylococcus aureus* α -hemolysin toxin, which preserves mitochondrial function and retains cytosolic proteins, while rendering the plasma membrane permeable to nucleotides and other small molecular weight compounds (Maechler *et al*, 1997). The K^+/H^+ ionophore nigericin was utilised to lower matrix pH under conditions of clamped pH_{cyto} (Nicholls, 2006). The effective concentration of nigericin was determined by monitoring mtAlpHi fluorescence in the permeabilised cells. The addition of the mitochondrial substrate succinate (1 mM) increased matrix pH from 7.04 to 7.4, yielding a ΔpH of 0.36 units, similar to the effect of glucose in intact cells. Nigericin at both 100 and 500 nM significantly reduced the matrix pH in the continued presence of 1 mM

succinate (Figure 8A and B). The same concentrations of nigericin further hyperpolarised the mitochondrial membrane potential as recorded with JC-1 when added after succinate stimulation (Figure 8C). DL- α -glycerolphosphate (effective concentration 5 mM), which directly provides

Figure 5 Comparison of glucose-evoked mitochondrial matrix alkalinisation, insulin secretion, mitochondrial calcium and cytosolic ATP rises in rat islets. Individual rat islets were analysed as described in Figure 2. (A) Average mitochondrial pH response. For clarity, the error bars ($N=7$) are displayed only every 120 s. Insulin secretion (B), mitochondrial calcium (C) and cytosolic ATP (D) were measured from groups of 100–150 islets. For mitochondrial calcium or ATP measurements, islets were infected with Ad-RIP-mtAequorin or Ad-RIP-Luciferase, respectively. Insulin samples were collected every 30 s from the efflux of mitochondrial calcium measurements.

Figure 6 Mitochondrial calcium signals play little or no role in mitochondrial matrix alkalinisation or hyperpolarisation of the electrical potential in β -cell mitochondria. **(A)** Intact rat islets expressing mtAlpHi were stimulated with 16.7 mM glucose in the absence of extracellular calcium (KRBH containing 16.7 mM glucose, 0 mM calcium chloride, 0.4 mM EGTA). As indicated, EGTA was then removed and 1.5 mM calcium chloride was added. **(B)** Glucose responses of the pH_{mito} in the absence of extracellular calcium. Values are presented as mean $\pm$ s.e.m. ($N = 4$). **(C)** Rat islets expressing mtAlpHi were stimulated with tolbutamide (500 μ M) followed by glucose (16.7 mM) in the continued presence of tolbutamide. **(D)** Ratiometric measurements of JC-1 fluorescence changes during glucose stimulation (Materials and methods). Dispersed rat islet cells were loaded with JC-1. Changes in fluorescence ratios from basal levels were normalised to ratio differences between resting and maximal depolarisation induced by 10 μ M FCCP and expressed as percent changes of the mitochondrial membrane potential. At the end of the trace, EGTA was removed and 1.5 mM calcium was added to the cells lacking extracellular calcium. Average glucose responses in the presence (black trace) or absence of extracellular calcium (grey trace; 0.4 mM EGTA) are shown. **(E)** Quantification of the ratio changes. **(F)** Rat islets expressing mtAlpHi were treated with 1 μ M rotenone and then stimulated with 16.7 mM glucose. **(G)** Average pH_{mito} responses of rat islets to glucose in the presence of 1 μ M rotenone.

Figure 7 Kinetic comparison of matrix alkalinisation and the increase of the mitochondrial electrical potential. INS-1E cells expressing mtAlpHi (**A**; $N=6$) or loaded with JC-1 (**B**; $N=7$) were stimulated with 16.7 mM glucose. For clarity, standard errors are included only every 120 s. FCCP (10 μ M) was used to depolarise the mitochondria.

reducing equivalents at complex II of the respiratory chain, caused a similar hyperpolarisation as 1 mM succinate (Figure 8D). Again, nigericin polarised the mitochondrial membrane potential further. This hyperpolarisation reflects a compensatory effect to the loss of Δ pH striving to maintain a constant electrochemical gradient across the inner mitochondrial membrane (Nicholls, 2006). Therefore, nigericin should only marginally affect the electrochemical potential and consequently the driving force on the ATP synthase. As a read-out of mitochondrial energy metabolism, we measured total ATP synthesised from ADP in the permeabilised cells. Succinate or α -glycerolphosphate increased ATP generation approximately five-fold (Figure 8E and F) when compared with control in the absence of exogenous nutrient. Nigericin strongly reduced substrate-induced ATP synthesis. Succinate-dependent ATP production, for instance, was reduced by more than 80% by 100 nM nigericin. Therefore, the pH gradient is a key determinant of mitochondrial substrate-mediated ATP synthesis.

Discussion

In the pancreatic β -cell, nutrients play a dual role, providing substrates as well as generating signals for the activation of mitochondria essential for insulin secretion. Here, we report that the pH_{mito} alkalinises during nutrient stimulation and suggest that variations of this parameter act as a regulator of mitochondrial metabolism, specifically ATP generation. Our

results using either ^{31}P -NMR or the pH-sensitive protein probe mtAlpHi show that pH_{mito} is not constant but is modulated by the metabolic state of the cell.

We suggest that nutrient stimulation of the β -cell causes an imbalance in proton handling of mitochondria, which results in the net export of protons from the matrix space. Proton export could result from influx of positive charge, for instance, in the form of ions, under conditions where the mitochondrial membrane potential would be maintained through the extrusion of protons by the respiratory chain. Continued influx of cations such as calcium may result in sufficient compensatory proton export to overcome the buffering capacity of the matrix space. Here we find that alkalinisation still occurs under conditions that prevent mitochondrial calcium influx. Nevertheless, removal of extracellular calcium decreased the mitochondrial alkalinisation response by 20%, arguing that net calcium accumulation in the mitochondria contributes marginally to the observed increase of pH_{mito} . A second possible mechanism is the net export of negatively charged metabolites. For instance, citrate is exported from the mitochondria together with protons (Joseph *et al*, 2006), which could contribute to matrix alkalinisation. Whether export of metabolites such as citrate contributes to matrix alkalinisation has not yet been tested. Subsequently, rates of proton-coupled transport are reduced, resulting in a new steady state. Alternatively, a new steady state may be reached as the sum of the electrical and chemical potentials becomes limited by the proton motive force that β -cell mitochondria are able to generate.

Mitochondrial alkalinisation to glucose was observed neither in HeLa cells (Llopis *et al*, 1998) nor in HepG2 cells (this study). Nutrient-dependent mitochondrial alkalinisation, therefore, seems to be specific for β -cells. This may be due to the fact that the β -cell is designed to sense nutrients, and secondly, because glycolysis-derived pyruvate is effectively funneled into the β -cell mitochondria for oxidative metabolism in this highly respiratory cell type (Sekine *et al*, 1994; Schuit *et al*, 1997). Other cell types that fulfill these criteria may exhibit similar nutrient-dependent pH_{mito} changes, a possibility that remains to be tested.

It is noteworthy that the oxidative substrate leucine mimicked the effect of glucose on β -cell pH_{mito} . On the other hand, the non-secretagogue substrate glutamine (Sener *et al*, 1981) did not alkalinise. The pronounced alkalinisation to nutrient secretagogues only, suggests that mitochondrial matrix alkalinisation could be an important metabolic event determining the β -cell secretory response.

A surprising finding of this study is the very low matrix pH value under resting conditions in INS-1 cells and rat islet β -cells. In a number of other cell types, including cultured primary cells and cell lines, mitochondrial matrix pH is alkaline, varying from pH 7.6 to 8.3 (Llopis *et al*, 1998; Matsuyama *et al*, 2000; Abad *et al*, 2004). As a consequence of the low pH_{mito} of the resting β -cell, the Δ pH is also small (0.13 pH units in rat islet cells). During glucose stimulation, the Δ pH of primary β -cell mitochondria increases markedly, reaching 0.51 pH units. Compared with the small pH_{cyto} changes in rat islets (0.08 pH units; see also Shepherd and Henquin, 1995; Stienet *et al*, 2007), the pH_{mito} increase is pronounced (0.45 pH units), yielding an increase of the Δ pH across the inner mitochondrial membrane of 0.37 pH units. Thereby, the proton chemical gradient adds -22 mV to the

Figure 8 Lowering of the pH_{mito} in permeabilised cells strongly reduces mitochondrial ATP synthesis. INS-1E cells were permeabilised with α -hemolysin toxin and analysed for mitochondrial matrix pH changes (**A**, **B**), mitochondrial membrane potential polarisation (**C**, **D**) and ATP generation (**E**, **F**). Mitochondria of the permeabilised cells were stimulated with either 1 mM succinate (**A**, **B**, **C**, **E**) or 10 mM DL- α -glycerolphosphate (**D**, **F**). (**A**) Nigericin caused acidification of the mitochondrial matrix at both 100 and 500 nM. (**B**) Quantification of the mitochondrial pH measurements ($N=4-7$). The same concentrations (**C**, **D** and data not shown) resulted in hyperpolarisation of the inner mitochondrial membrane ($N=6-8$). Nigericin strongly reduced ATP generation from both 1 mM succinate (**E**) and 10 mM DL- α -glycerolphosphate (**F**) oxidation ($N=3-8$). Values are presented as mean $\pm$ s.e.m.

proton electrochemical potential, a significant contribution to the hyperpolarisation of the inner mitochondrial membrane by glucose.

The mitochondrial matrix alkalinisation to glucose is relatively slow. Comparison with the kinetics of first- and second-phase insulin secretion would argue that alkalinisation is principally important during the second phase. No correlation was observed between the rapid initial rise of the mitochondrial calcium signal and matrix alkalinisation. Mitochondrial calcium signals were not necessary for the observed alkalinisation during glucose stimulation, nor was calcium alone sufficient to raise the mitochondrial matrix pH. Calcium removal slows glucose metabolism (Hellman *et al*, 1974), which could explain the small attenuation of matrix alkalinisation.

The halftime of the mitochondrial alkalinisation is also slower than the increase of the electrical potential. Extrusion

of few protons from the matrix space is sufficient to build up a membrane potential, while resulting in little alkalinisation due to the high proton-buffering capacity of the mitochondrial matrix. Alkalinisation ensues as the net proton export overcomes mitochondrial buffering. However, the pH increase may be counteracted by the formation of protons from oxidative metabolism. Considering the many aspects of mitochondrial metabolism that affect pH_{mito} without impacting on the electrical potential, these kinetic differences are not unexpected.

Alkalinisation correlates quite closely with the net increase of cytosolic ATP. Similarly, oxygen consumption was found to increase for about 15 min after an initial rapid rise in respiration (Hutton and Malaisse, 1980). The respiratory chain is the key driving force for the maintenance of the ΔpH across the inner mitochondrial membrane of the β -cell, as inhibition of complex I resulted in a rapid acidification of the matrix space.

These results also confirm the close correlation between mitochondrial ATP synthesis and alkalinisation.

The ^{31}P -NMR saturation transfer experiments substantiate the link between mitochondrial alkalinisation and ATP synthesis during glucose stimulation. The results obtained for pH_{mito} are in good agreement with mtAlpHi recordings. Remarkably, the acceleration of ATP synthesis as measured by ^{31}P -NMR was higher in the mitochondrial compared with the cytosolic compartment. Consequently, the relative contribution of mitochondria to ATP synthesis increases at stimulatory glucose concentrations. To our knowledge, this is the first study reporting pH_{mito} measurements with ^{31}P -NMR as well as mitochondrial unidirectional rate constants for ATP synthesis and ATP synthesis rates *in situ*.

The association between matrix alkalinisation and mitochondrial ATP synthesis was further corroborated in α -toxin permeabilised INS-1E cells by direct manipulation of the pH_{mito} . In the presence of the K^+/H^+ ionophore nigericin, substrate-mediated alkalinisation was strongly attenuated, accompanied by the expected hyperpolarisation of the electrical potential maintaining the driving force on the ATP synthase. This manoeuvre caused a dramatic reduction of ATP synthesis both for succinate, which must be taken up by mitochondria, and for α -glycerolphosphate, which directly transfers electrons to the respiratory chain. These findings underline the importance of the pH_{mito} and/or the ΔpH in mitochondrial ATP generation. Low pH_{mito} has also been implicated in the inhibition of ATP synthesis in isolated mitochondria from cardiomyocytes (Rouslin, 1987).

To date, only mitochondrial calcium has been suggested to serve as a signal linking nutrient stimulation to the metabolic activation of mitochondria (for a review, see Wiederkehr and Wollheim, 2008). Our work points to the importance of mitochondrial matrix alkalinisation in this process. We propose that the increased ΔpH ensures the sustained uptake of pyruvate and Pi for oxidative metabolism and ATP synthesis during sustained glucose-dependent insulin secretion. This study puts mitochondrial matrix pH on the map as a signal associated with metabolic activation.

Materials and methods

Most chemicals and reagents used for the experiments were from Sigma and Fluka Chemie (Switzerland). Coelenterazine was purchased from Calbiochem. Beetle luciferin was obtained from Promega.

Rat islet isolation and culture conditions

Islets were isolated from 200 to 300 g male Wistar rats (Janvier, France) by collagenase (Roche Diagnostics, Switzerland) digestion as described (Franklin *et al*, 2005). Islets were cultured in a humidified atmosphere containing 5% CO_2 in RPMI-1640 medium (Invitrogen, Switzerland) containing 11 mM glucose, 10 mM Hepes, 10% heat-inactivated fetal calf serum (Brunschwig AG, Switzerland), 50 $\mu\text{g}/\text{ml}$ penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 100 $\mu\text{g}/\text{ml}$ gentamicin (Essex Chemie AG, Switzerland) (islet medium).

Cell culture conditions

INS-1E cells were cultured in RPMI 1640 medium containing 11 mM glucose (Invitrogen, Switzerland) supplemented with 10 mM Hepes (pH 7.3), 10% (v/v) heat-inactivated fetal calf serum (Brunschwig AG, Switzerland), 50 μM β -mercaptoethanol, 1 mM sodium pyruvate, 50 $\mu\text{g}/\text{ml}$ penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (INS medium). HepG2 cells were grown in DMEM medium containing 5.6 mM glucose, 4 mM L-glutamine and 1 mM sodium pyruvate (Invitrogen, Switzerland) and 10% (v/v) heat-inactivated fetal calf serum.

Intracellular pH measurements using ^{31}P -NMR phosphate chemical shifts

INS-1 cell pellets were re-suspended in a 3% alginate solution in PBS (w/v). Beads of ~ 1 –2 mm diameter were formed by extruding the slurry from a 28-gauge needle dropping into a 1.1% BaCl_2 solution. The cells were allowed to recover overnight in a spinner flask containing INS medium. Alginate-entrapped INS-1 cells were perfused in the bioreactor in the bore of the AVANCE-500 NMR spectrometer, and ^{31}P -NMR spectra and saturation transfer experiments were acquired during step changes of metabolic substrates. ^{31}P -NMR spectra were continuously collected in 20-min experiments (3–4 measurements for each substrate level). The samples were oxygenated, and physiological pH and temperature were maintained.

The chemical shift of Pi by ^{31}P -NMR has been shown to be pH dependent, and the differences in the Pi frequency from the α -phosphate resonance of ATP (α -ATP) can be used to determine intracellular pH values after calibration (Gadian *et al*, 1979; Petroff *et al*, 1985). A calibration curve of Pi chemical shift frequencies with respect to pH was determined with Pi in buffered salt solutions.

ATP synthesis rate measurements

Changes in the intensity of Pi as measured by ^{31}P -NMR saturation transfer techniques are indicative of and proportional to the rate of ATP synthesis by that cellular pool of Pi. ATP synthesis rates are calculated as $k^{-1} \times [\text{Pi}]_{\text{in}}$, where k^{-1} is the rate constant, k (min^{-1}) = $\delta M/M_0 \times 1/T_1$, and $[\text{Pi}]_{\text{in}}$ is the intracellular concentration of the metabolically active Pi pool. $\delta M/M_0$ is the change in the intracellular Pi signal when the terminal phosphate of ATP is saturated compared with the Pi signal with a saturation pulse offset an equal distance downfield from the Pi resonance ($\delta M/M_0 = (M_0 - M_z)/M_0$). T_1 was measured using standard inversion-recovery experiments in separate studies. T_1 for extracellular Pi was calculated to be 4.13 s and T_1 for intracellular Pi was calculated to be 1.58 s at basal glucose. The intracellular Pi concentration was calculated from the relative signal intensities of the extracellular (Pi_{ext}) and intracellular (Pi_{in}) Pi.

Recombinant adenoviruses

The adenoviruses expressing mitochondrially targeted Aequorin (Ad-RIP-mitoAequorin) or cytoplasmic Luciferase (Ad-RIP-Luciferase) were constructed as described (Ishihara *et al*, 2003; Brun *et al*, 2004). The mtAlpHi DNA was cloned into the pTRE-Shuttle vector and re-cloned into the Adeno-X virus plasmid backbone for doxycycline-inducible expression (CLONTECH Laboratories). The tetON virus carrying the transcriptional regulator was provided with the expression system from CLONTECH Laboratories. Adenoviruses were amplified in HEK293 cells.

Virus infection protocols

At 1 day after plating, cultured cells were infected for 90 min at 37°C. After 2–3 days, the cells were analysed. Freshly isolated rat islets were recovered for 2 h in islet medium and were then infected for 90 min. For perfusion experiments, 100–150 infected rat islets were placed onto A431 extracellular-matrix-coated Thermanox cover slips (15 mm diameter, Nalge Nunc, USA) (Ishihara *et al*, 2003). For cytosolic and pH_{mito} measurements, 20 islets were placed in the centre of a matrix-coated 25 mm glass coverslip (Menzel GmbH, Germany).

Luminescence measurements

Mitochondrial calcium and cytosolic ATP measurements were performed as described (Kennedy *et al*, 1996; Ishihara *et al*, 2003). Measurements were performed in Krebs-Ringer bicarbonate Hepes buffer (KRBH): 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4 , 0.5 mM MgSO_4 , 1.5 mM CaCl_2 , 10 mM Hepes, 5 mM NaHCO_3 , 2.5 mM glucose pH 7.4.

Single-cell imaging

Expression of the mtAlpHi gene was induced with 1 $\mu\text{g}/\text{ml}$ of doxycycline after infection with the Ad-tetON-mtAlpHi and Ad-tetON regulatory virus. mtAlpHi measurements on attached rat islets were performed 2–3 days after infection. Titration of the pH_{mito} was performed as described (Abad *et al*, 2004). Image acquisition was performed on an inverted microscope (Zeiss Axiovert 200M, Carl Zeiss AG, Switzerland), with an array laser

confocal spinning disk (QLC100, VisiTech, UK). Cells were imaged using a 63 × (numerical aperture 1.4) oil-immersion objective (Carl Zeiss AG) and excited using 488 nm laser light. The emission wavelength was 535 nm. Images were acquired every 10 s and were analysed using Metafluor 6.3 Software (Universal Imaging, Molecular Devices Corporation, USA).

For pH_{cyto} measurements, attached INS-1E or rat islets were washed once in KRBH and loaded with the pH indicator BCECF (1 μM; Molecular Probes) for 8 min at 37°C. The pH_{cyto} was measured ratiometrically exciting with laser light of 440/488 nm and measuring the emission at 535 nm in KRBH containing sulfinpyrazone (100 μM).

Membrane potential measurements

Cell suspensions from isolated rat islets were prepared as described (Franklin *et al*, 2005). Dispersed islet cells from about 50 rat islets were seeded in black-walled 96-well plates (Greiner bio-one GmbH, Germany). Before the measurement, the cells were transferred for 1 h to islet medium (4 mM glucose). The cells were washed with KRBH 0.1% bovine serum albumin (fraction V, AppliChem, Germany) and incubated for 30 min at 37°C in the presence of 350 nM JC-1 (Invitrogen, Switzerland). They were then washed twice with KRBH 0.1% bovine serum albumin and incubated for 20 min. JC-1 fluorescence was measured ratiometrically at 37°C. The wavelengths used were 490 nm excitation/540 nm emission (green; monomer) and 540 nm excitation/590 nm emission (red; J-aggregates) in a multi-well fluorescence reader (FlexStation, Molecular Devices Corporation, USA) as described (Park *et al*, 2008).

INS-1E cell permeabilisation with α-hemolysin toxin

INS-1E cells were washed with Ca²⁺-free KRBH and then permeabilised with 1 μg/ml of *Staphylococcus aureus* α-hemolysin toxin (Sigma) in intracellular buffer (140 mM KCl, 5 mM NaCl, 7 mM MgSO₄, 1 mM KH₂PO₄, 20 mM HEPES, 10.2 mM EGTA,

1.65 mM CaCl₂, pH 7.0 titrated with KOH) for 10 min at 37°C (Maechler *et al*, 1997). After the permeabilisation step, the cells were washed with intracellular buffer containing 0.5% BSA before incubation in intracellular buffer supplemented with 1 mM ATP (no BSA) (or 10 μM ADP when ATP synthesis was monitored). pH_{mito} was recorded in intracellular buffer solution but was measured otherwise as described for intact cells. ATP production was measured after 15 min incubation of INS-1E cells in 48-well plates (2 × 10⁵ cells/well) using a bioluminescence assay kit (HS II, Roche Diagnostics, Switzerland). Measurement of the mitochondrial membrane potential was performed in 96-well plates (5 × 10⁴ cells/well), and cells were loaded with JC-1 and monitored in an intracellular buffer solution containing 1 μM JC-1.

Insulin assay

Insulin was measured during mitochondrial calcium recordings in the effluent of the perfusion chamber every 30 s. Insulin concentrations were determined using an enzyme immunoassay (SPI bio, France).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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