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Mitochondrial matrix pH controls oxidative phosphorylation and metabolism-secretion coupling in INS-1E clonal β cells

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ABSTRACT **Glucose-evoked mitochondrial signals augment ATP synthesis in the pancreatic β cell. This activation of energy metabolism increases the cytosolic ATP/ADP ratio, which stimulates plasma membrane electrical activity and insulin granule exocytosis. We have recently demonstrated that matrix pH increases during nutrient stimulation of the pancreatic β cell. Here, we have tested whether mitochondrial matrix pH controls oxidative phosphorylation and metabolism-secretion coupling in the rat β-cell line INS-1E. Acidification of the mitochondrial matrix pH by nigericin blunted nutrient-dependent respiratory and ATP responses (continuously monitored in intact cells). Using electrophysiology and single cell imaging, we find that the associated defects in energy metabolism suppress glucose-stimulated plasma membrane electrical activity and cytosolic calcium transients. The same parameters were unaffected after direct stimulation of electrical activity with tolbutamide, which bypasses mitochondrial function. Furthermore, lowered matrix pH strongly inhibited sustained, but not first-phase, insulin secretion. Our results demonstrate that the matrix pH exerts a control function on oxidative phosphorylation in intact cells and that this mode of regulation is of physiological relevance for the generation of downstream signals leading to insulin granule exocytosis. We propose that matrix pH serves a novel signaling role in sustained cell activation.—Akhmedov, D., Braun, M., Mataki, C., Park, K.-S., Pozzan, T., Schoonjans, K., Rorsman, P., Wollheim, C. B., Wiederkehr, A. Mitochondrial matrix pH controls oxidative phosphorylation and metabolism-secretion coupling in INS-1E clonal β cells. *FASEB J.* 24, 4613–4626 (2010). www.fasebj.org**

Key Words: ATP · insulin · respiration · mtAlpHi/YC3.6

THE PANCREATIC β CELL is able to sense a large number of secretagogues to adapt insulin release to blood nutrient concentrations. Nutrients such as glucose or amino acids are sensed *via* their uptake and metabolism, which results in downstream signals that lead to insulin granule exocytosis. Mitochondria play a central role in this metabolism-secretion coupling. They are required for the oxidative metabolism of nutrients and oxidative phosphorylation. The resulting increase of the ATP/ADP ratio causes closure of K_{ATP} channels, thereby initiating β-cell electrical activity (triggering pathway). Other mitochondria-derived metabolites may potentiate exocytosis by mechanisms that act in parallel with the regulation of the K_{ATP} channel (amplifying pathway) (1–3). Therefore, inhibition of mitochondrial function, in particular, respiratory chain activity, impairs insulin secretion (4–6).

The respiratory chain oxidizes NADH and $FADH_2$ derived from oxidative metabolism to reduce molecular oxygen, which is coupled to the establishment of a proton electrochemical gradient across the inner mitochondrial membrane that drives the mitochondrial ATP synthase. The electrochemical potential is the sum of the electrical potential across the inner mitochondrial membrane and a chemical component proportional to the pH difference between the mitochondrial matrix and the cytosol (7).

Glucose stimulation of the β cell results in the activation of a large number of energy-demanding processes, including plasma membrane electrical activity, associated ion handling, Ca^{2+} cycling, gene transcription/translation, and insulin granule transport, as well as exocytosis. β-Cell mitochondria are able to cope with this increased energy demand by adjusting oxygen consumption and ATP synthesis rates (4, 8–10). Rapid adjustment of ATP synthesis to energy demand has been proposed to depend on a feedback control mech-

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anism, whereby an increase in ADP and inorganic phosphate (P_i) due to ATP hydrolysis accelerates mitochondrial respiration to maintain a normal ATP/ADP*P_i ratio (phosphorylation potential). This mode of regulation may not necessarily apply to all tissues. For instance, in the heart, the energy demand increases severalfold during enhanced workload. Nevertheless, no changes of ATP or ADP levels during physiological stimulation were observed (reviewed in ref. 11).

In the pancreatic β cell, the phosphorylation potential increases during nutrient stimulation despite elevated energy consumption (9, 12, 13). This is achieved through a rise in oxygen consumption and ATP synthesis rates (8, 10). As ADP levels following nutrient activation are reduced (12, 14, 15), a feedback control mechanism for the acceleration of mitochondrial oxidative phosphorylation is unlikely. Therefore, other signals are needed to coordinate cytosolic energy demand and mitochondrial ATP synthesis. For instance, Ca^{2+} signals exert such a function, as they are relayed into mitochondria, where they activate matrix dehydrogenases, as well as the ATP synthase (16–18).

We have recently identified matrix pH as a potential signal that, like Ca^{2+}, could link nutrient stimulation to β-cell energy metabolism (10). Matrix pH has been studied in a number of cell types *in situ* and ranges from 7.7 to 8.2 (19–21). Matrix pH is therefore ~1 pH unit more alkaline than the cytosolic pH, which is close to 7. This proton concentration gradient adds about −60 mV to the electrochemical potential (about −150 mV) across the inner mitochondrial membrane (22), thus significantly contributing to the driving force on the ATP synthase. The proton chemical gradient is also linked to a number of transport processes exchanging metabolites or ions between mitochondria and the cytosol (23, 24).

We have previously shown that in INS-1E cells (clonal β cells) and primary rat β cells, the matrix pH is unusually low but alkalinizes following nutrient stimulation, paralleling the net cytosolic ATP increase (10). Furthermore, we demonstrated that preventing matrix alkalinization in permeabilized INS-1E cells using the ionophore nigericin abolished substrate-driven ATP synthesis from exogenously added ADP (10). On the basis of these findings, we have tested here whether the mitochondrial pH is a modulator of oxidative phosphorylation and metabolism-secretion coupling in intact INS-1E cells.

MATERIALS AND METHODS

Most chemicals and reagents used for the experiments were from Sigma (Buchs, Switzerland) and Fluka Chemie (Buchs, Switzerland). Coelenterazine was purchased from Calbiochem (La Jolla, CA, USA). Beetle luciferin was obtained from Promega (Basel, Switzerland). BCECF and JC-1 were from Molecular Probes (Invitrogen, Basel, Switzerland). BSA fraction V was from AppliChem (Darmstadt, Germany).

Cell culture conditions

INS-1E cells were cultured in RPMI 1640 medium containing 11 mM glucose (Invitrogen) supplemented with 10 mM HEPES (pH 7.3), 10% (vol/vol) heat-inactivated FCS (Brunschwig AG, Basel, Switzerland), 50 μM β-mercaptoethanol, 1 mM sodium pyruvate, 50 μg/ml penicillin, and 100 μg/ml streptomycin (INS medium).

Recombinant adenoviruses

The adenoviruses expressing mitochondrially targeted aequorin (Ad-RIP-mitoAequorin), cytoplasmic luciferase (Ad-RIP-Luciferase), and the Ad-RIP-mtAlpHi were constructed as described previously (10, 25). For cytosolic Ca^{2+} measurements, the Ca^{2+}-responsive protein construct YC3.6 (26) was cloned behind the rat insulin promoter. This construct was recloned into an adenovirus vector backbone (Clontech Laboratories, Mountain View, CA, USA). Adenoviruses were amplified in HEK293 cells.

Luminescence measurements

One day after plating, cultured cells were infected for 90 min at 37°C with either Ad-RIP-luciferase or Ad-RIP-mitoAequorin and analyzed 1 or 2 d later. All measurements described in this study 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 $NaHCO_3$, and 2.5 mM glucose, pH 7.4. The cells were perifused at a rate of 1 ml/min.

Single-cell imaging

For cytosolic and mitochondrial pH measurements, 10^6 INS-1E cells were plated onto polyornithine-coated 25-mm glass coverslips (Menzel, Bielefeld, Germany) in a 6-well plate. One day after plating, the INS-1E cells were infected with Ad-RIP-mtAlpHi (10) or Ad-RIP-YC3.6. Titration of the mitochondrial pH was performed as described previously (20). Image acquisition was performed on an inverted microscope (Zeiss Axiovert 200M; Carl Zeiss AG, Zurich, Switzerland) with an array laser confocal spinning disk (QLC100; VisiTech, Sunderland, UK). Cells were imaged using a ×63 (numerical aperture 1.4) oil-immersion objective (Carl Zeiss AG). For mitochondrial pH measurements, mtAlpHi was excited using 488-nm laser light. The emission wavelength was 535 nm. Images were acquired every 10 s and analyzed using Metafluor 6.3 software (Universal Imaging; Molecular Devices, Sunnyvale, CA, USA). For cytosolic Ca^{2+} measurements, YC3.6 was excited with 440-nm light, and emission was followed at 480 and 535 nm. Images were acquired every 2 s.

For cytosolic pH measurements, INS-1E cells were washed once in KRBH and loaded with the pH indicator BCECF (1 μM) for 8 min at 37°C. The cytosolic pH was measured ratiometrically, exciting with laser light of 440/488 nm and measuring the emission at 535 nm in KRBH containing sulfinpyrazone (100 μM).

Mitochondrial membrane potential measurements

INS-1E cells were plated into black-walled polyornithine-coated 96-well plates (75,000 cells/well; Greiner Bio-One,

Frickenhausen, Germany). Cells were washed with KRBH and 0.1% BSA and incubated for 30 min at 37°C in the presence of 350 nM JC-1 (Invitrogen). The cells were washed twice with KRBH and 0.1% BSA 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 multiwell fluorescence reader (FlexStation; Molecular Devices) as described previously (27).

Oxygen consumption

INS-1E cells were seeded onto polyornithine-coated plates (Seahorse Biosciences, North Billerica, MA, USA). The cells were grown for 2 or 3 d in INS medium, reaching ~80% confluence (10^5 cells/well). The cells were washed 2 times with KRBH and 2.5 mM glucose. The plates were placed into the Seahorse XF24 instrument, and oxygen consumption rates were determined every 9 min. Stock solutions of glucose or nigericin were added during the run and immediately mixed to reach final concentrations as indicated in the figures.

Electrophysiology

Electrophysiological measurements were performed using the perforated-patch whole-cell configuration of the patch-clamp technique. Patch pipettes were pulled from borosilicate glass and fire-polished (tip resistance 4–8 MΩ). Recordings were performed using an EPC-10 amplifier and Pulse software (HEKA Electronics, Malone Bay, NS, Canada). The temperature in the recording chamber was kept at 32–33°C by continuous superfusion with heated KRBH. The pipette solution consisted of (mM) 76 K_2SO_4, 10 KCl, 10 NaCl, 1 $MgCl_2$, and 5 HEPES (pH adjusted to 7.35 with KOH). Electrical contact was established by the addition of the pore-forming antibiotic amphotericin B (0.24 mg/ml) to the pipette solution.

Cell loss and apoptosis measurements

INS-1E cells were incubated in KRBH and 16.7 mM glucose with or without 500 nM nigericin. After incubation, the cells were washed with phosphate-buffered saline and detached by trypsinization. Cell numbers were determined using a counting chamber (Neubauer, Marienfeld, Germany).

Apoptosis was measured using a cell death detection $ELISA^{PLUS}$ kit (Hoffman-La Roche, Basel, Switzerland).

Insulin secretion

INS-1E cells were plated into polyornithine-coated 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA; 4×10^5 cells/well) and grown for 48 h. Prior to the experiments, the cells were washed 3 times with KRBH, 2.5 mM glucose, and 0.1% BSA. The cells were preincubated for 30 min at 37°C in the same buffer. Before incubation, the wells were washed once with KRBH, 2.5 mM glucose, and 0.1% BSA and incubated for 30 min in the presence of different stimuli. Supernatants were saved for insulin measurements. Cells were extracted in acid ethanol overnight at 4°C to determine the insulin content. Insulin was measured using an enzyme immunoassay kit (SPI bio, Massy, France). For kinetic secretion measurements, 0.5-ml insulin samples were collected every 30 s in polystyrol tubes (PSN-55; Millian, Basel, Switzerland) containing BSA (final concentration 0.05%) during perifusion experiments also continuously recording the mitochondrial Ca^{2+} signal.

Static ATP and ADP measurements

INS-1E cells were prepared as for insulin secretion measurements. ATP was determined using a bioluminescence kit (HS II; Roche Diagnostics, Rotkreuz, Switzerland). ADP was measured after enzymatic removal of ATP, as described previously (28).

RESULTS

During glucose stimulation, mitochondrial matrix pH in the insulin-secreting INS-1E cells increased from pH 7.25 ± 0.04 to 7.78 ± 0.02 (n=5) (**Fig. 1*A***), consistent with our earlier findings (10). Mitochondrial pH was determined using the pH-sensitive probe mtAlpHi, as described previously (10, 20). The resting matrix pH was only 0.19 pH units higher than the cytosolic pH determined under identical conditions using the fluorescent indicator BCECF (Fig. 1*C*). Fifteen minutes after initiation of the glucose response, the ΔpH increased 3.5-fold to 0.66 (Fig. 1*C*). To assess the role of mitochondrial matrix pH and the alkalinization associated with glucose stimulation, we have used nigericin. This ionophore mediates the electroneutral exchange of potassium and protons across biological membranes. Matrix pH was lowered rapidly by nigericin (500 nM) and partially recovered over the following 15 min (Fig. 1*B*, *D*). In the presence of nigericin, matrix pH remained 0.31–0.49 pH units lower than the control along the entire time course of the glucose response.

Matrix acidification lowers ΔpH and causes hyperpolarization of the mitochondrial electrical potential

Nigericin is not specific for the inner mitochondrial membrane but also acts on other cellular membranes (22). For instance, at the plasma membrane, nigericin may mediate the export of potassium down its concentration gradient in exchange for the uptake of protons, and as a consequence cause acidification of the cytosol. However, 500 nM nigericin did not alter the cytosolic pH under conditions that caused a pronounced acidification of the mitochondrial matrix (Fig. 1*C*, *D*). Thus, the ΔpH across the inner mitochondrial membrane collapsed shortly after nigericin addition and partially recovered thereafter (Fig. 1*D*). At the concentration applied here, nigericin lowers the ΔpH across the inner mitochondrial membrane by ~0.4 pH units throughout.

Mitochondria attempt to maintain their electrochemical gradient constant. A decrease of the mitochondrial matrix pH of 0.4 pH units (equivalent to −24

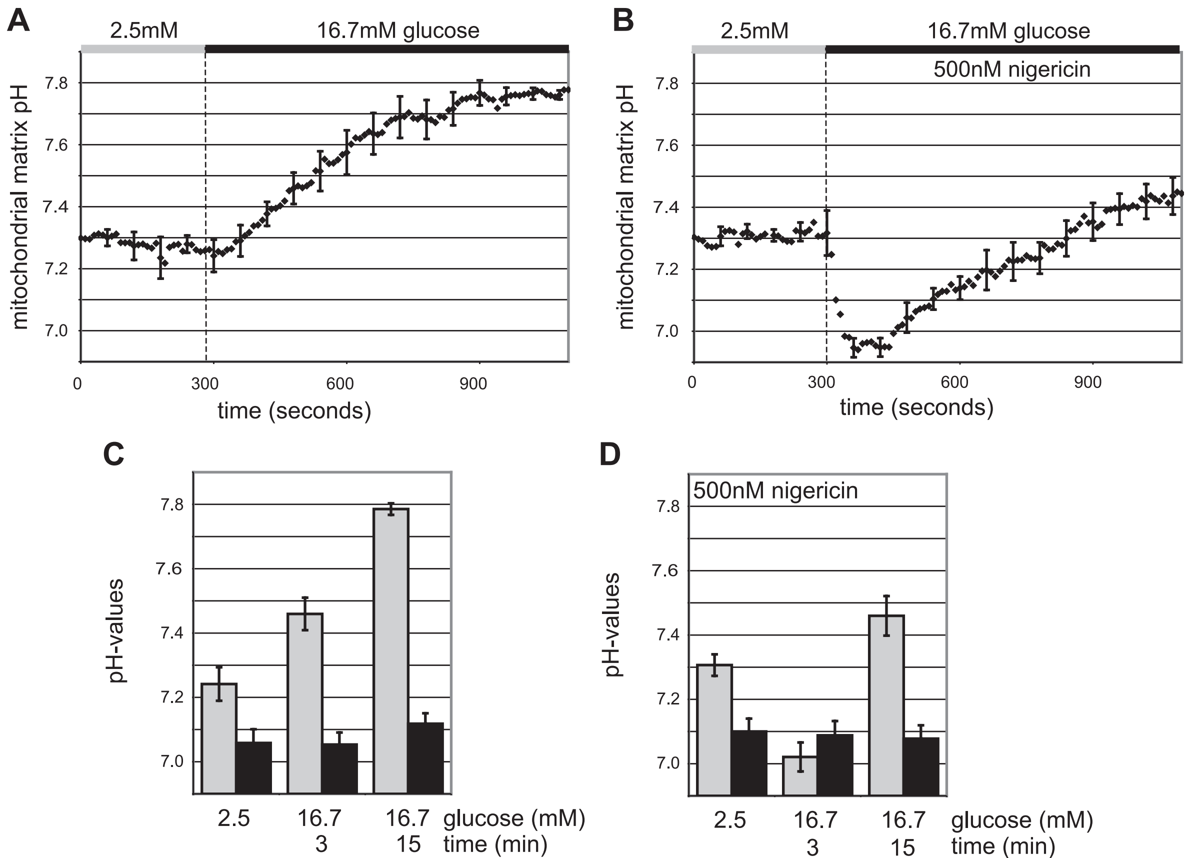

Figure 1. Nigericin lowers mitochondrial but not cytosolic pH in INS-1E cells. INS-1E cells were shifted from 2.5 to 16.7 mM glucose (control; *A*, *C*) or to 16.7 mM glucose buffer containing 500 nM nigericin (*B*, *D*). *A*, *B*) Mitochondrial matrix pH was measured using mtAlpHi; $n = 5$. For clarity, SE is shown only every 60 s. *C*, *D*). Cytosolic pH was determined using the ratiometric fluorescent dye BCECF (solid bars; n=5) and compared to the corresponding mitochondrial pH values (shaded bars; n=5). Values are mean ± SE pH glucose response. For all panels, pH values were determined using titration curves, as described in Materials and Methods.

mV of proton motive force) as observed here should therefore be compensated for by an increase of the electrical potential. To study the electrical potential, we used the ratiometric probe JC-1 (27). Consistent with earlier studies, glucose stimulation of INS-1E cells causes hyperpolarization of the inner mitochondrial membrane (**Fig. 2*A***) (27, 29). Nigericin further hyperpolarized the mitochondrial electrical gradient in a concentration-dependent manner (Fig. 2*A*). Hyperpolarization by the ionophore was also observed when cells were maintained continuously in the presence of 2.5 mM glucose (Fig. 2*B*). Such compensatory hyperpolarization of the electrical gradient across the inner mitochondrial membrane in response to nigericin has been described previously in cerebellar granule neurons (22). Unlike the protonophore FCCP, nigericin does not cause uncoupling of the inner mitochondrial membrane (Fig. 2*A*, *B*).

Matrix acidification inhibits the normal ATP response to glucose

The compensatory hyperpolarization should maintain the driving force on the ATP-synthase, thereby preserving ATP homeostasis. Therefore, we tested whether matrix acidification affects ATP synthesis in response to glucose. Relative changes of ATP were measured kinetically by expressing luciferase in the cytosol of INS-1E cells (25) (Fig. 2*C*). Following glucose stimulation, the cytosolic ATP signal increased. This glucose-stimulated response was strongly reduced by nigericin at both 100 and 500 nM (Fig. 2*D*). During the first 5 min of the glucose response, a reduction of the matrix pH had little or no effect. Thereafter, ATP levels steadily increased in the control but were strongly attenuated when the matrix pH was lowered by nigericin (Fig. 2*C*). Under basal glucose conditions, manipulation of the matrix pH with 500 nM nigericin caused a rapid reduction of the cytosolic ATP levels during the first 3 min (Fig. 2*E*). After 20 min, 500 nM had lowered the cytosolic ATP levels by 17.3 ± 1% (Fig. 2*E*; n=3). Thus, the matrix pH or ΔpH is relevant for maintenance of ATP homeostasis, as well as glucose-stimulated net ATP changes in INS-1E cells.

The effect of nigericin was further studied by measuring total cellular ATP and ADP after extraction of INS-1E cells 5 and 20 min following stimulation with 16.7 mM glucose (Fig. 2*F*). After 5 min, 16.7 mM glucose significantly elevated the ATP/ADP ratio com-

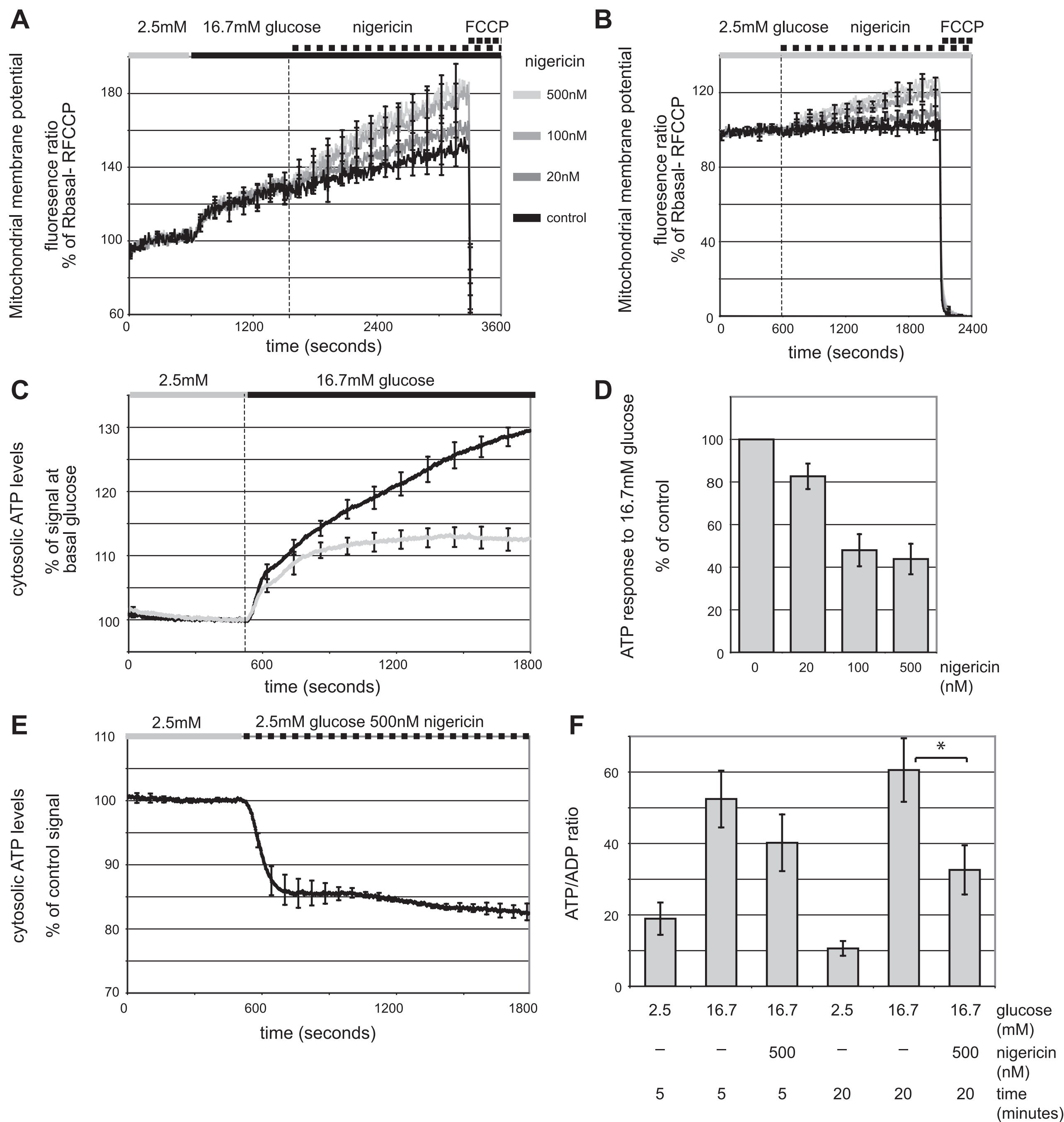

Figure 2. Matrix acidification causes hyperpolarization of the inner mitochondrial membrane and reduces glucose-dependent cytosolic ATP rises. *A*, *B*) INS-1E cells were loaded for 30 min with 350 nM of JC-1. Hyperpolarization was expressed as an increase of the JC-1 fluorescence ratio over basal (100%). Ratio after depolarization with FCCP (10 μM) was set to 0. INS-1E cells were stimulated with 16.7 mM glucose (*A*) or maintained at 2.5 mM glucose (*B*). Nigericin was added at increasing concentrations (20 nM, 100 nM, 500 nM), indicated by different grayscale levels. Control responses are shown in black. Dashed line indicates the time point of nigericin addition (n=3; each experiment performed in duplicate). Error bars = SE; displayed every 120 s. *C*) Relative cytosolic ATP changes were monitored in INS-1E cells 2 d after infection with Ad-RIP-luciferase. Net ATP changes were monitored during glucose stimulation in the presence (gray trace) or absence (black control trace) of 500 nM nigericin (n = 5). Error bars = SE; displayed every 120 s. *D*) ATP responses were quantified after 20 min in the presence of 16.7 mM glucose and increasing concentrations of nigericin. Data are expressed as mean ± SE percentage of control response to glucose performed on the same day (n=4). *E*) ATP changes were measured as shown in *C*. INS-1E cells were perifused with KRBH containing 2.5 mM glucose; 500 nmol was added, as indicated by the dashed line (n=3). Error bars = SE; displayed every 120 s. *F*) For static measurement of the ATP/ADP ratio, INS-1E cells were incubated for 5 and 20 min at the indicated glucose concentrations in the presence or absence of nigericin. ATP and ADP concentrations in lysates were determined and expressed as the ATP/ADP ratio (n=4). Values are means ± SE. *$P < 0.05$.

pared to the cells maintained at 2.5 mM glucose. At this early time point, the ATP/ADP response had a tendency to be lower in the presence of nigericin, but the difference was not significant. At 20 min, nigericin had lowered the glucose-stimulated increase of the ATP/ADP ratio by >50% (Fig. 2*F*).

Matrix acidification inhibits accelerated oxygen consumption in response to glucose

The presented results are in agreement with the interpretation that matrix acidification slows mitochondrial ATP synthesis despite hyperpolarization of the electrical potential across the inner mitochondrial membrane. Through its effect on other biological membranes, nigericin may also lower cytosolic ATP, for instance, by stimulating ATP-hydrolyzing membrane proteins, such as the Na^+/K^+ ATPase or the proton pumping vacuolar ATPase. To test whether matrix pH indeed affects mitochondrial energy metabolism, we studied respiration after manipulation of the mitochondrial pH. In control INS-1E cells, glucose caused a rapid increase in oxygen consumption. At 9 min after exposure to 16.7 mM, glucose respiration was already significantly increased, from a basal rate of 228 ± 4.4 pmol/min/10^5 cells (basal respiration) to 531 ± 26.2 pmol/min/10^5 cells. This initial rapid increase was followed by a slower phase reaching 837 ± 71 pmol/min/10^5 cells 45 min after initiation of the glucose stimulus (**Fig. 3*A***). The kinetics of the respiratory responses resembles the net changes of ATP as described in Fig. 2*C*.

The initial rapid glucose-dependent acceleration of respiration was unaffected by nigericin. In contrast, the slower second phase of the respiratory response to glucose was strongly reduced or even abolished when 100 or 500 nM nigericin was added at the time of glucose addition (Fig. 3*A*). To ensure that the initial increase in respiratory rate is unaffected by the matrix pH, we performed similar glucose-dependent oxygen consumption experiments after preincubation with nigericin. The result confirms that nigericin is unable to suppress the early effect of glucose on respiration (Fig. 3*B*). The data can therefore not be explained by a putative slow action of nigericin on mitochondrial function. Under basal conditions (2.5 mM glucose), nigericin caused little or no decrease of the oxygen consumption rate (Fig. 3*B* and data not shown).

We conclude that matrix pH is a key regulator of mitochondrial oxidative phosphorylation. As alkalinization of the matrix pH occurs during glucose stimulation, this may be of particular importance for the second phase of the net ATP (Fig. 2*C*) and respiratory response (Fig. 3*A*, *B*) to glucose.

Using alternative substrates to stimulate mitochondrial respiration, we narrowed the possible control sites modulated by matrix pH. Leucine is a nutrient secretagogue that is metabolized to form acetyl-CoA without a direct involvement of glycolysis or pyruvate dehydrogenase. Leucine increases the net ATP levels (Fig. 3*C*) and causes matrix alkalinization similar to glucose (10). Leucine-dependent induction of oxygen consumption was rapid, reaching a maximal value at 9 min, slowly decreasing thereafter (Fig. 3*D*). Nigericin lowered the amplitude of the respiratory response by 41.4 ± 9.0% and abolished the leucine-dependent net ATP increase.

Methyl succinate is membrane permeable and can be used in intact cells to stimulate respiration. Oxidation of this substrate to fumarate by complex II provides reducing equivalents in the form of FADH2 to the respiratory chain. Direct stimulation of INS-1E cell by methyl succinate caused a sharp rise of respiration and cytosolic ATP levels. Both responses were blunted by 500 nM nigericin (Fig. 3*E*, *F*). The result with methyl succinate argues that matrix pH regulates oxidative phosphorylation downstream of complex II.

Effect of nigericin on mitochondrial functions in HeLa and HepG2 cells

To assess the general importance of this regulatory mechanism in the control of oxidative phosphorylation, we also tested the effect of nigericin on mitochondrial functions on two transformed widely used cell lines, HeLa and HepG2 (**Fig. 4**). To augment the contribution of mitochondria to total ATP synthesis in HeLa cells, glucose was omitted from the KRBH and replaced by 1 mM lactate and 0.1 mM pyruvate. Nigericin hyperpolarized the mitochondrial electrical potential in HeLa cells (Fig. 4*A*), similar to the results obtained with INS-1E cells (Fig. 2*A*, *B*). The effect of nigericin on cytosolic ATP levels was, however, weak (reduced by 5.9±3.0% after 30-min incubation in the presence of 500 nM nigericin; Fig. 4*C*). Nigericin (500 nM) also inhibited respiration of HeLa cells, but this was only apparent during prolonged incubation with the ionophore (Fig. 4*B*). In HepG2 cells, respiration was not affected by nigericin (Fig. 4*D*). We conclude that mitochondrial functions of INS-1E cells are particularly sensitive to the manipulation of the matrix pH by nigericin.

Nigericin does not result in cell death or mitochondrial fragmentation

The observed strong effects on respiration and ATP changes in INS-1E cells could be due to cell loss or death. To distinguish this possibility from a true effect of the matrix pH changes on mitochondrial respiration, cell numbers were determined at the end of a static incubation. One- or 2-h incubations in the presence of nigericin (500 nM) did not lower the number of cells when compared to control (KRBH and 16.7 mM glucose; **Fig. 5*A***). A majority of cells was lost when

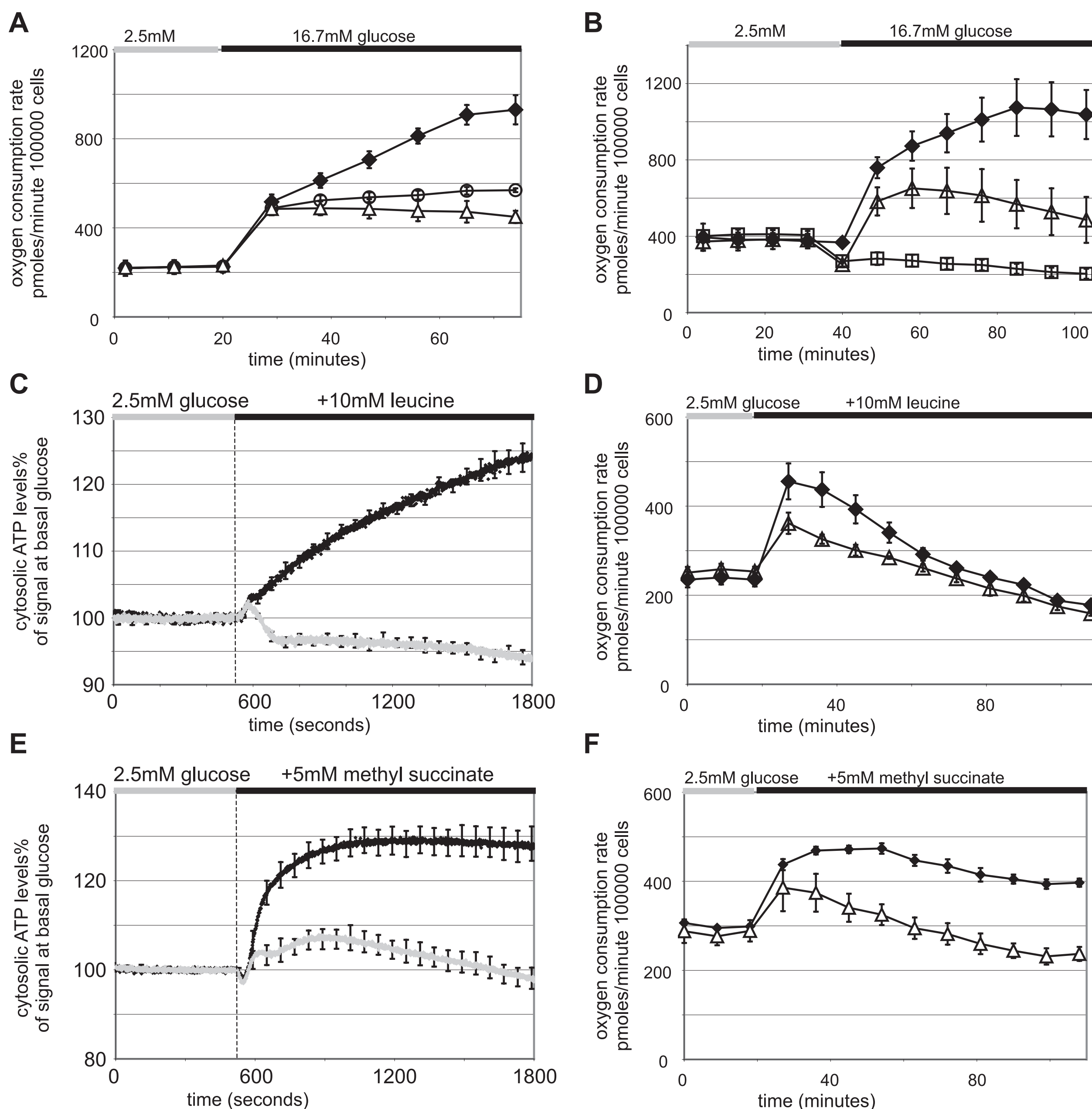

Figure 3. Nigericin inhibits substrate-dependent acceleration of mitochondrial respiration in intact INS-1E cells. INS-1E cells were stimulated with 16.7 mM glucose (*A*, *B*), 10 mM leucine (*C*, *D*), or 5 mM methyl succinate (*E*, *F*). Oxygen consumption rates (*A*, *B*, *D*, *F*) were measured every 9 min before and after substrate stimulation. ATP changes (*C*, *E*) were monitored as described in Fig. 2*D*. Nigericin was added at the same time as the indicated nutrient except in *B*, where nigericin (500 nM) was added 9 min prior to the initiation of the glucose response (open triangles). At the same time point, nigericin was also added to cells maintained at 2.5 mM glucose (*B*; open squares). For respiration experiments, SE is shown for each condition and time point. *A*, *B*) Measurements were performed in quadruplicate in 3 independent experiments (n=3; n=12). *C*, *E*) Error bars = SE; shown every 60 s (n = 3). *D*, *F*) Measurements were performed in quadruplicate; values are means ± SE (n=4). Qualitatively similar results were obtained in 3 independent experiments. Solid diamonds, control; open triangles, 500 nM nigericin; open circles, 100 nM nigericin.

hydrogen peroxide (0.1 or 1 mM) was added as a positive control.

As a readout for apoptosis, we quantified cytosolic histone-bound DNA fragments using a cell-death detection kit (see Materials and Methods). Under control conditions, the number of apoptotic cells is low. Longer incubations in KRBH and 16.7 mM glucose resulted in doubling of cells undergoing apoptotic cell death, but at no time point did nigericin significantly enhance apoptosis (Fig. 5*B*). Furthermore, no histone-bound DNA fragments were detected in the cell supernatant, arguing against nigericin-induced necrotic cell death (data not shown).

Furthermore, mitochondria remained filamentous and retained JC-1 when incubated in KRBH and 16.7 mM glucose containing 500 nM nigericin (Fig. 5*C*). In

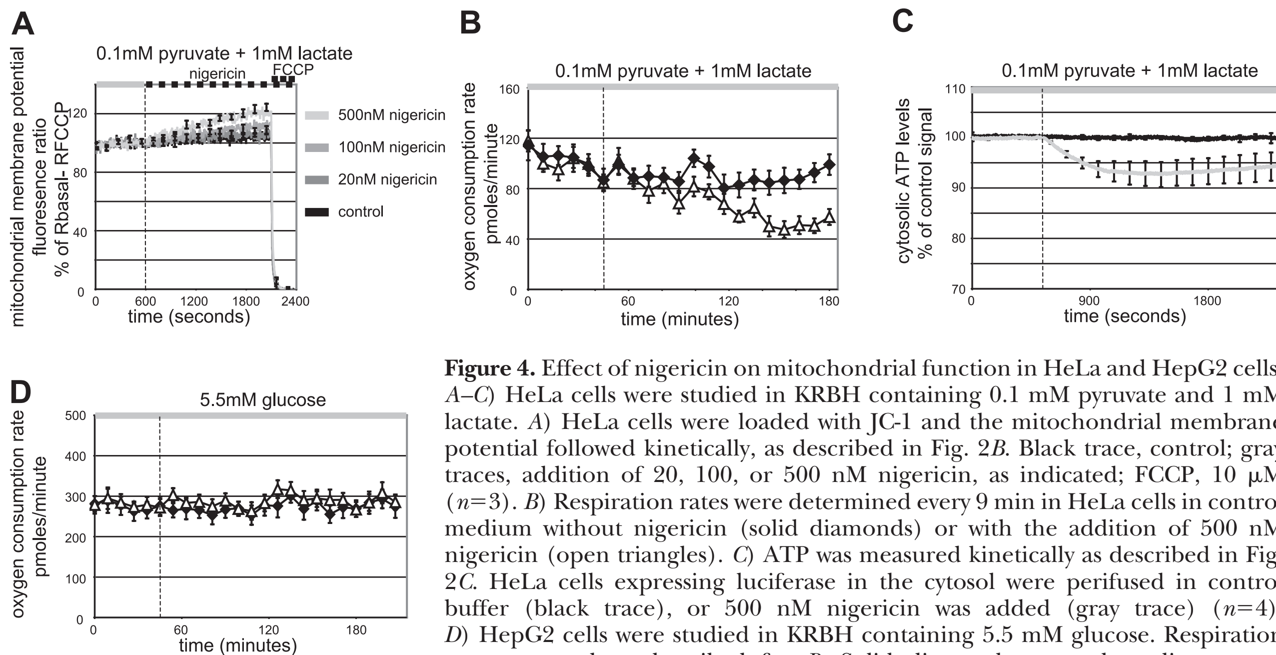

Figure 4. Effect of nigericin on mitochondrial function in HeLa and HepG2 cells. *A–C*) HeLa cells were studied in KRBH containing 0.1 mM pyruvate and 1 mM lactate. *A*) HeLa cells were loaded with JC-1 and the mitochondrial membrane potential followed kinetically, as described in Fig. 2*B*. Black trace, control; gray traces, addition of 20, 100, or 500 nM nigericin, as indicated; FCCP, 10 μM (n=3). *B*) Respiration rates were determined every 9 min in HeLa cells in control medium without nigericin (solid diamonds) or with the addition of 500 nM nigericin (open triangles). *C*) ATP was measured kinetically as described in Fig. 2*C*. HeLa cells expressing luciferase in the cytosol were perifused in control buffer (black trace), or 500 nM nigericin was added (gray trace) (n=4). *D*) HepG2 cells were studied in KRBH containing 5.5 mM glucose. Respiration was measured as described for *B*. Solid diamonds, control medium; open triangles, 500 nM nigericin (n=4). In all panels, vertical dashed lines indicate time of nigericin addition. Values are means ± SE.

contrast, hydrogen peroxide induced mitochondrial fragmentation and partial loss of the dye (Fig. 5*C* and data not shown).

Most important, hyperpolarization of the inner mitochondrial membrane was more pronounced in the presence of nigericin even when analyzed for extended incubation times (Fig. 5*D*; 90 min). Mitochondrial integrity and cell viability were not compromised by nigericin over the time course studied here.

Nigericin suppresses plasma membrane electrical activity and thereby cytosolic Ca^{2+} signals

A main goal of this study was to assess whether matrix pH changes and the associated regulation of mitochondrial function is of physiological importance for metabolism-secretion coupling. We therefore tested whether the inhibitory effect of nigericin on cytosolic ATP levels is sufficient to blunt electrical activity resulting from closure of the K_{ATP} channels. To this end, we monitored the electrical activity of INS-1E cells using perforated-patch membrane potential recordings (30). In this recording configuration, electrical activity is controlled by endogenously synthesized ATP. Stimulation of resting INS-1E cells with 16.7 mM glucose caused plasma membrane depolarization and initiated the firing of action potentials (**Fig. 6*A*, *B***). Nigericin dramatically decreased the frequency of glucose-induced action potentials (Fig. 6*A*, *C*) and partially repolarized the cell (Fig. 6*B*). Nigericin does not appear to have an acute effect on the membrane potential, as action potential frequency started to decrease only after a delay of 1–3 min, which was also associated with a partial membrane hyperpolarization (Fig. 6*A*, *B*). Lowering matrix pH increased plasma membrane K_{ATP} conductance (Fig. 6*D*, *E*). This is likely the consequence of lowered cytosolic ATP levels after nigericin treatment (Fig. 2*C*, *D*, *F*). Therefore, the link between mitochondrial activation and the plasma membrane electrical activity is perturbed by nigericin.

As an additional kinetic readout for a defect in metabolism secretion coupling, we monitored cytosolic Ca^{2+} transients, using the ratiometric probe YC3.6 (26). The YC3.6 coding sequence was cloned into an adenovirus vector backbone under the control of the rat insulin promoter. Following infection with the Ad-RIP-YC3.6, groups of INS-1E cells (6–12 cells) expressing the Ca^{2+}-sensitive protein were assessed for their ability to respond to glucose (**Fig. 7*A***). Stimulation with 16.7 mM glucose induced Ca^{2+} transients in 81% of the cells. Glucose increased the frequency of Ca^{2+} transients 5.1-fold over the frequency observed at resting glucose concentrations, whereas the average amplitude of the Ca^{2+} transients was only slightly increased (Fig. 7*B*, *C*). After the addition of nigericin to glucose-activated INS-1E cells, the frequency of cytosolic Ca^{2+} transients decreased almost back to basal (Fig. 7*A*, *B*). This attenuation was reversible (Fig. 7*A*).

Tolbutamide, a blocker of the K_{ATP} channel, was able to initiate action potentials and thereby cytosolic Ca^{2+} transients, even in the presence of nigericin (Figs. 6*A*, *C* and 7*D*). These results argue that nigericin does not suppress action potentials or Ca^{2+} influx by a direct effect but indeed acts by altering mitochondrial ATP synthesis and regulation of the K_{ATP} channel conductance. Taken together, the electrophysiological recordings and cytosolic Ca^{2+} measurements demonstrate a very strong inhibition of the K_{ATP}-channel-dependent

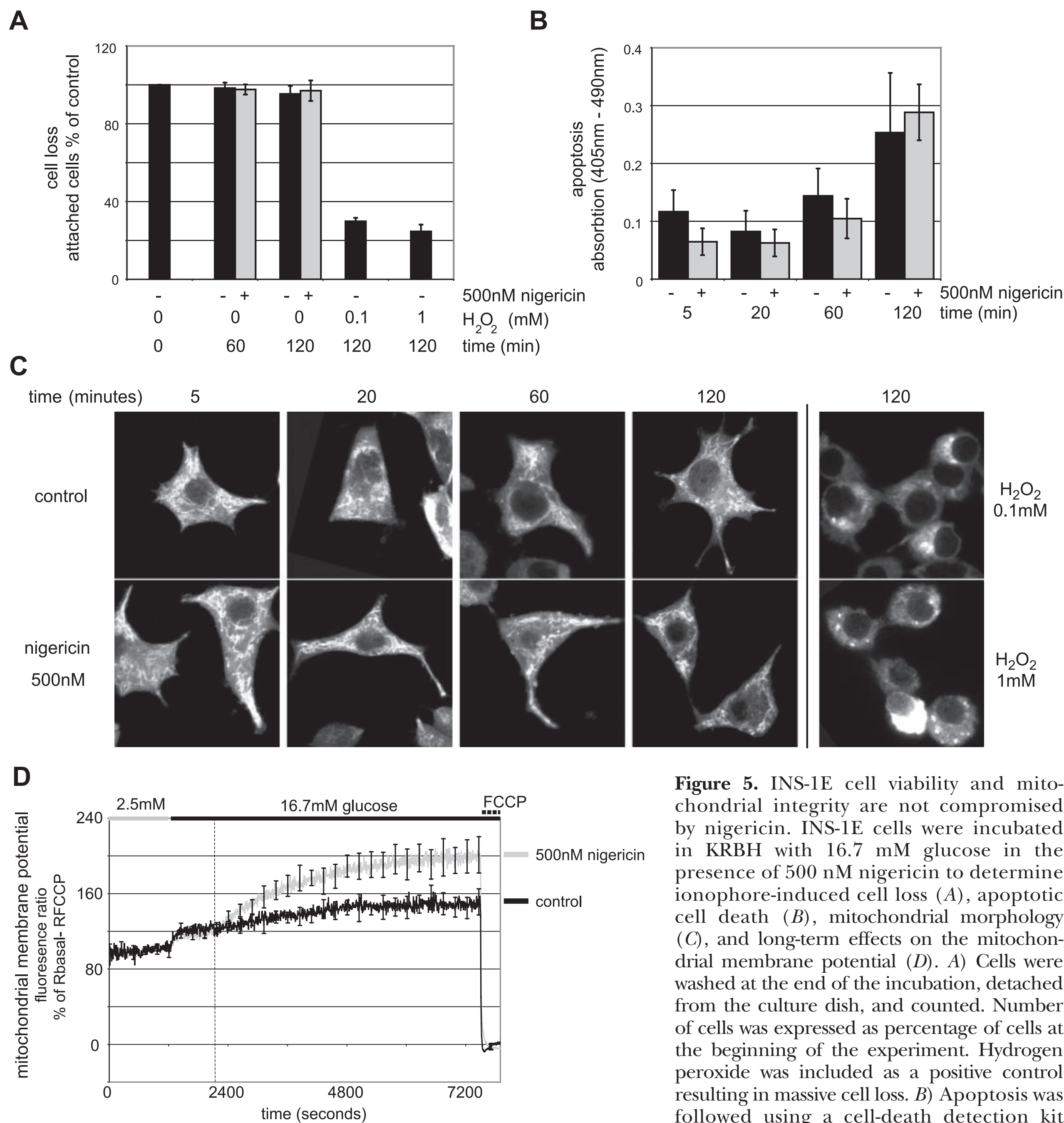

Figure 5. INS-1E cell viability and mitochondrial integrity are not compromised by nigericin. INS-1E cells were incubated in KRBH with 16.7 mM glucose in the presence of 500 nM nigericin to determine ionophore-induced cell loss (*A*), apoptotic cell death (*B*), mitochondrial morphology (*C*), and long-term effects on the mitochondrial membrane potential (*D*). *A*) Cells were washed at the end of the incubation, detached from the culture dish, and counted. Number of cells was expressed as percentage of cells at the beginning of the experiment. Hydrogen peroxide was included as a positive control resulting in massive cell loss. *B*) Apoptosis was followed using a cell-death detection kit measuring cytosolic histone-bound DNA fragments. *C*) Localization of JC-1 dye in mitochondria of INS-1E cells incubated for the indicated times in control KRBH 16.7 mM glucose or also containing nigericin or hydrogen peroxide. *D*) Mitochondrial membrane potential was measured as described in Fig. 2*A*. Cells were kept in KRBH containing 16.7 mM glucose (control; black trace) or 16.7 mM glucose and 500 nM nigericin for 90 min (gray trace) (n=3) Error bars = SE; for clarity, shown only every 240 s.

pathway of glucose-stimulated insulin secretion when the mitochondrial matrix pH was manipulated with nigericin.

Nigericin strongly reduces insulin secretion from INS-1E cells

This marked effect on metabolism-secretion coupling was also revealed in static insulin secretion experiments. In control INS-1E cells, 16.7 mM glucose raised insulin secretion 5.6-fold. Basal insulin secretion was not reduced by nigericin, but glucose-stimulated insulin secretion was only 1.6-fold over basal when the ionophore was present (**Fig. 8*A***).

Given the pronounced suppression of insulin secretion by nigericin, we further tested whether the ionophore interferes directly with the exocytotic machinery, in addition to its upstream attenuation of mitochondrial function. To address this, we used

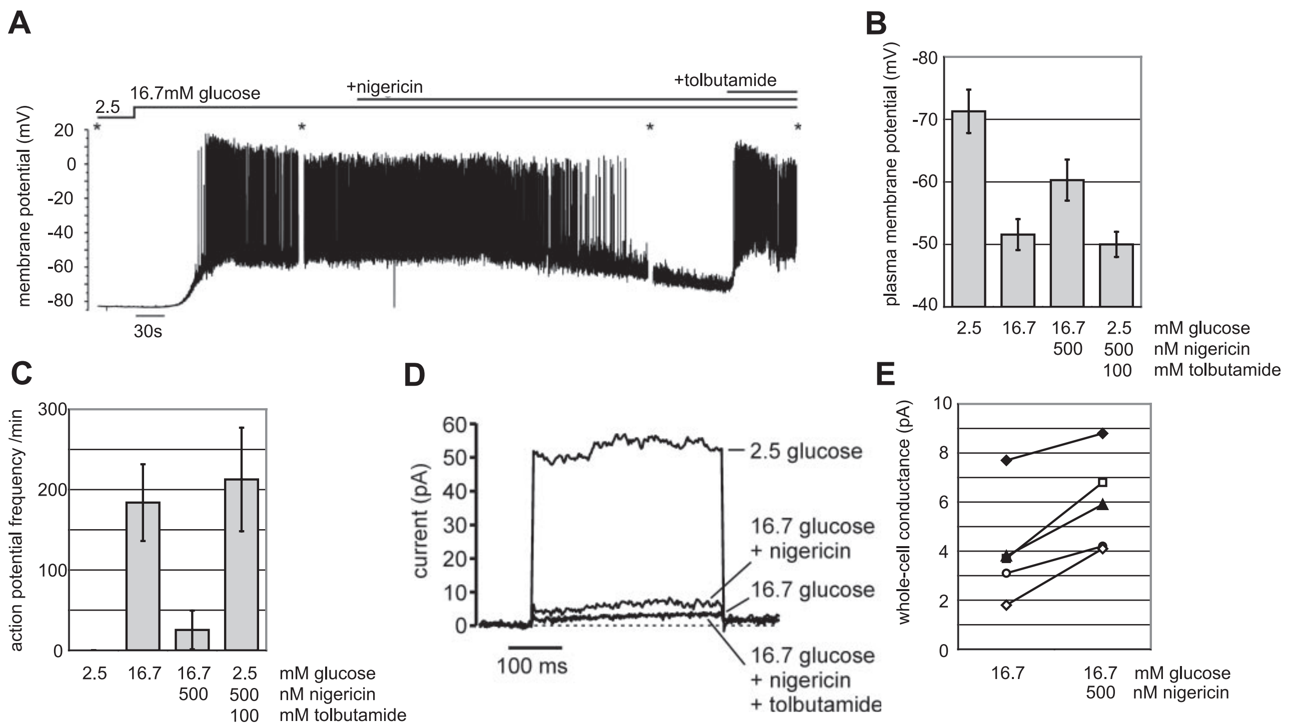

Figure 6. Nigericin arrests glucose-dependent action potentials in INS-1E cells. Plasma membrane electrical activity was studied in INS-1E cells using the perforated-patch technique. *A*) INS-1E cell displaying action potentials in response to glucose and inhibition by 500 nM nigericin. Tolbutamide reinitiates action potentials despite the continued presence of nigericin. Cell was switched to voltage clamp at the times indicated by the asterisks. *B*, *C*) Quantification of the interspike plasma membrane potential (*B*) and action potential frequencies (*C*) of 7 INS-1E cells. Values are means ± SE. *D*) Whole-cell conductance measurement of cell shown in *A* using a 350-ms depolarization from −70 to −60 mV. *E*) Results from 5 cells, comparing whole-cell conductance at 16.7 mM glucose with and without nigericin.

phorbol 12-myristate 13-acetate to stimulate PKC and thereby proteins of the exocytotic machinery (31). The addition of the phorbol ester neither increased mitochondrial pH nor affected cytosolic ATP levels (data not shown). We therefore conclude that it stimulates insulin secretion independently of mitochondrial activation. Nigericin did not affect phorbol 12-myristate 13-acetate-stimulated insulin secretion (Fig. 8*B*). This experiment demonstrates that nigericin does not interfere with the function of the exocytotic machinery *per se*.

The measurements of net cytosolic ATP levels (Fig. 2*C*, *F*) and mitochondrial respiration (Fig. 3) indicate that the initial phase of glucose-dependent mitochondrial activation is unaffected when the mitochondrial matrix is acidified. We, therefore, tested whether this early activation of mitochondria is sufficient to initiate metabolism-secretion coupling. For this, we followed the mitochondrial Ca^{2+} signal in a population of INS-1E cells after infection with an adenovirus expressing mitochondrially targeted aequorin. Mitochondrial Ca^{2+} rises are closely correlated with the cytosolic Ca^{2+} signals, and therefore faithfully report on the kinetics of β-cell activation. During these experiments, samples were also taken from the efflux of the perifusion to determine the effect of nigericin on the kinetics of insulin secretion. Following glucose stimulation, Ca^{2+} rose from 170 nM to a maximal concentration of 950 nM in the mitochondrial matrix (Fig. 8*C*). Stimulation with glucose in the presence of 500 nM nigericin resulted in an initial mitochondrial Ca^{2+} rise, similar to the control (Fig. 8*C* and data not shown). However, when nigericin was present, the mitochondrial Ca^{2+} signal was of much shorter duration than the control response, as the signal rapidly returned to basal (Fig. 8*C*). Perifusion with nigericin prior to the glucose stimulus did not prevent this transient mitochondrial Ca^{2+} rise, demonstrating that nigericin was not simply acting too slowly to prevent the early phase of the matrix Ca^{2+} signaling (data not shown). We conclude that initiation of metabolism-secretion coupling, and therefore, the initial mitochondrial Ca^{2+} rise to glucose is resistant to the acidification of the mitochondrial matrix pH. During these perifusion experiments, control INS-1E cells augmented insulin secretion ~5-fold when stimulated with 16.7 mM glucose, and insulin secretion remained elevated thereafter (Fig. 8*D*). In contrast, after acidification of the mitochondrial pH by nigericin, glucose-dependent insulin secretion was only transiently stimulated, peaked 2.5 min after initiation of the glucose stimulus, and then rapidly returned to basal rates (Fig. 8*D*). We conclude that the ΔpH across the inner mitochondrial membrane or the matrix pH has a very strong effect on metabolism-secretion coupling and becomes increasingly important during sustained-phase insulin secretion.

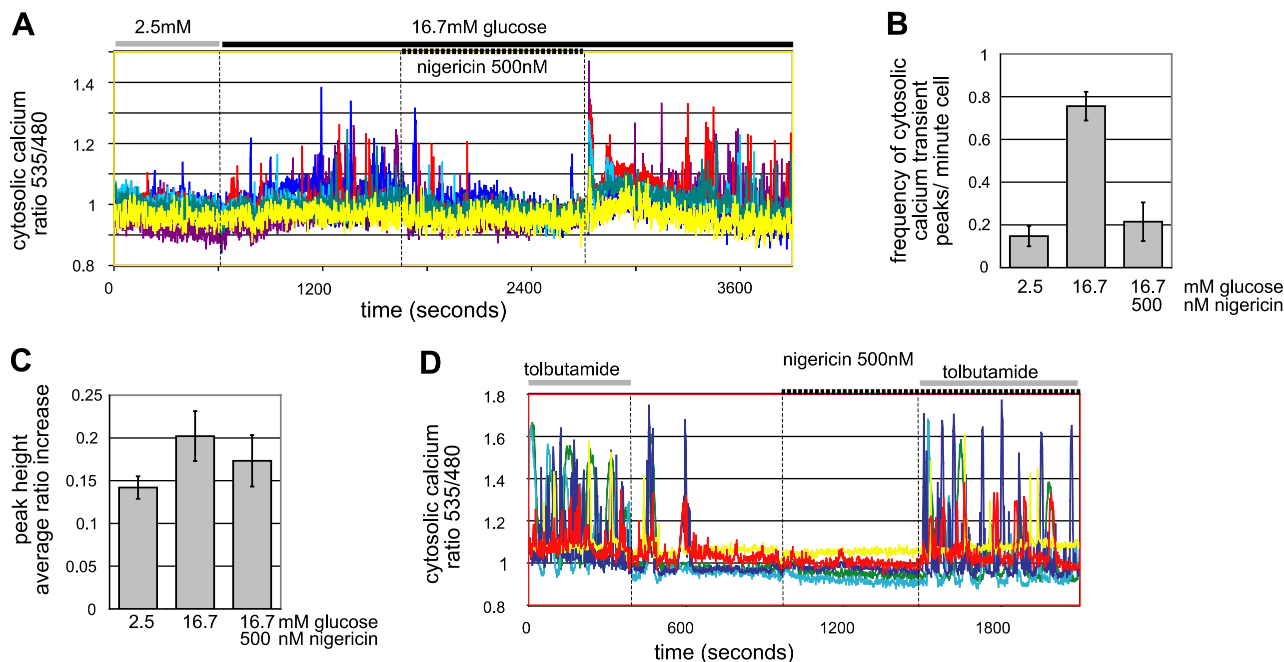

Figure 7. Lowering of the mitochondrial matrix pH blocks glucose- but not tolbutamide-dependent cytosolic Ca^{2+} transients. INS-1E cells were infected with Ad-RIP-YC3.6. At 2 d after infection, groups of cells expressing the transgene were analyzed. YC3.6 was excited with laser light of 440 nm, and emission was measured at 480 and 535 nm. A Ca^{2+} transient is reported as an increase of the 535/480-nm emission ratio. *A*) Ca^{2+} responses from 8 cells from a single experiment. Glucose (16.7 mM) and nigericin (500 nM) were added and removed as indicated. *B*, *C*) Quantification of the Ca^{2+} transient frequency (*B*) and the amplitude height (*C*) after stimulation with glucose in the presence or absence of nigericin. Forty-eight cells from 5 independent experiments were analyzed. *D*) Nigericin does not suppress tolbutamide-induced Ca^{2+} transients. Responses of 5 cells from a single experiment are shown.

DISCUSSION

In the pancreatic β cell, the ATP/ADP ratio acts as an essential upstream signal for insulin granule exocytosis (3). This signaling role of the ATP/ADP ratio may explain why β-cell energy metabolism must prevent a rise in ADP even during times of enhanced ATP hydrolysis (9, 12, 13). In this cell type, feedback activation of oxidative phosphorylation by ADP is therefore not a likely mechanism to explain nutrient stimulation of the mitochondrial ATP synthesis rate. Recently, we proposed that the mitochondrial matrix pH contributes to the control of mitochondrial ATP synthesis in β cells. This concept is based on our findings that matrix pH is low in the resting β cell (pH 7.25) and increases to pH 7.7 following stimulation with nutrient secretagogues (10). By contrast, in a large number of other cell types, matrix pH *in situ* was shown to be alkaline (close to pH 8) (10, 19–21). Furthermore, our earlier results demonstrate that preventing matrix alkalinization in permeabilized INS-1E cells almost completely abolished ATP synthesis from exogenous ADP (10).

In the present study, we have manipulated the mitochondrial matrix pH in intact insulin-secreting cells, in order to test whether mitochondrial pH regulates oxygen consumption and ATP synthesis and to assess whether the matrix pH has an effect on metabolism-secretion coupling and hormone release. For this purpose, we have used nigericin, which mediates the electroneutral exchange of protons against potassium across biological membranes. Nigericin caused a rapid acidification of the mitochondrial matrix pH driven by the pH gradient across the inner mitochondrial membrane. We point out that weak acids failed to cause matrix acidification, probably because of the high buffering capacity and pH-controlling mechanisms in the cytosol (data not shown). Nigericin (500 nM) caused mitochondrial acidification starting within the first 10 s after addition but, remarkably, had no acute effects on the cytosolic pH or the plasma membrane potential. Partial repolarization of the plasma membrane, cessation of action potentials, and cytosolic Ca^{2+} transients were only observed after 1–3 min following the addition of the compound. We conclude that these actions of nigericin are mainly a secondary consequence of the inhibition of mitochondrial function. By lowering the mitochondrial ATP response to glucose, nigericin increases the K_{ATP} channel conductance, which prevents plasma membrane depolarization, Ca^{2+} influx, and insulin granule exocytosis.

On average, nigericin lowered the ΔpH across the inner mitochondrial membrane by 0.4 pH units, thereby decreasing the electrochemical gradient, the driving force of the ATP synthase. This reduction was at least partially compensated by a hyperpolarization of the electrical gradient across the mitochondrial membrane. Despite this hyperpolarization, we observed a pronounced reduction of the cytosolic ATP,

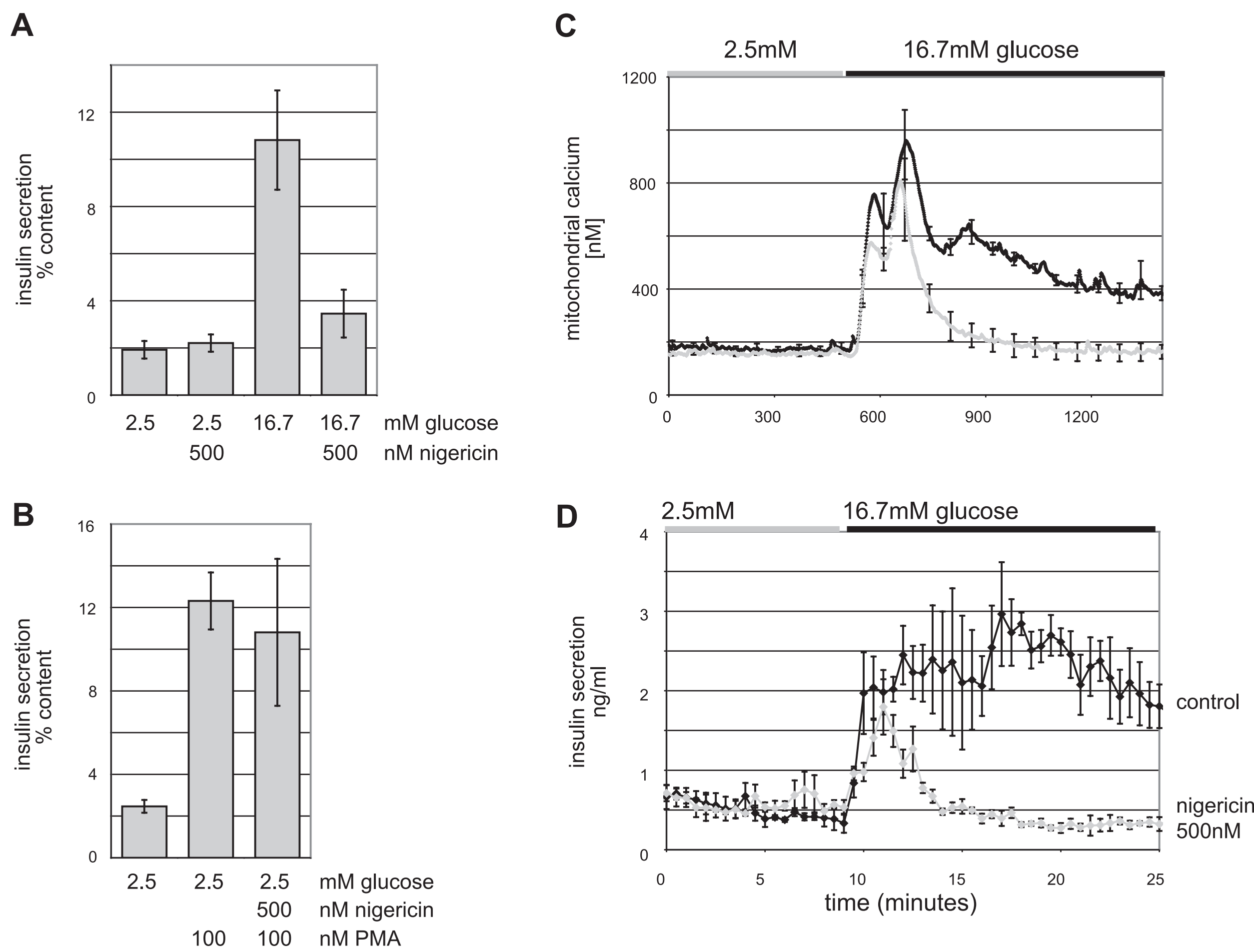

Figure 8. Mitochondrial matrix acidification blunts insulin secretion in INS-1E cells. *A*, *B*) INS-1E cells were incubated for 30 min in KRBH buffer containing glucose (2.5 or 16.7 mM; *A*), nigericin (500 nM), or PMA (100 nM; *B*) as indicated. Insulin was measured in the supernatant and the cell content after 30 min of incubation and expressed as percentage of content. *C*) INS-1E cells were infected with an adenovirus for the expression of mitochondrial aequorin 2 d prior to the experiment. After loading with coelenterazine, cells were perifused in KRBH and 2.5 mM glucose and stimulated with 16.7 mM glucose (black traces) or 16.7 mM glucose and 500 nM nigericin (gray traces). Mitochondrial aequorin-derived luminescence was monitored and recalculated to Ca^{2+} concentrations. *D*) Samples in the efflux of the mitochondrial Ca^{2+} measurements were taken every 30 s, and insulin concentration was determined. Flux rate was 1 ml/min. Error bars = SE (n=3).

as well as ATP/ADP ratio, in response to glucose. This blunting is likely due to inhibition of mitochondrial function rather than accelerated ATP hydrolysis in the cytosol, a conclusion that is supported by the inhibition of mitochondrial oxygen consumption by nigericin. Our data demonstrate that matrix pH or the linked ΔpH effectively controls oxidative phosphorylation in insulin-secreting cells.

Glucose caused a very pronounced time-dependent increase of the respiration rate in INS-1E cells, which appeared to be biphasic. A rapid initial acceleration of oxygen consumption was followed by a slower gradual rise. Interestingly, the initial increase of the oxygen consumption rate and ATP response to glucose were unchanged and slightly reduced, respectively, when the matrix pH was lowered. In contrast, the slower second phase leading to a gradual acceleration of mitochondrial energy metabolism depends on matrix alkalinization and possibly other mitochondrial signals. Similarly, nigericin strongly reduced leucine- and methyl succinate-stimulated mitochondrial energy metabolism. Both of these substrates mimic the effects of glucose on insulin secretion. These substrates were even more sensitive to matrix acidification than glucose, as illustrated by the more rapid onset of inhibition. The pronounced attenuation of oxidative phosphorylation after stimulation with methyl succinate suggests that the main control function by matrix pH is exerted downstream of complex II.

ΔpH is also important as a driving force for a number of metabolite transport steps across the inner mitochondrial membrane (24). For instance, the uptake of the energy substrates pyruvate and glutamate or the uptake of inorganic phosphate for ATP synthesis is coupled to the net import of protons, and therefore depends on ΔpH. Alkalinization of the mitochondrial matrix in the β cell is therefore a plausible mechanism to ensure continued provision of these substrates during sustained insulin secretion.

On the basis of our findings, we propose that matrix pH acts together with matrix Ca^{2+} to stimulate mitochondrial energy metabolism. Ca^{2+} has been shown to accelerate oxidative metabolism by activating matrix dehydrogenases (16). Matrix pH, on the other hand, may control respiration and ATP synthesis rates. Matrix Ca^{2+} and pH may not only complement each other but also directly depend on each other. However, our results in β cells argue against this possibility. First, the time courses of glucose-stimulated increases in mitochondrial Ca^{2+} and pH are strikingly different. Second, raising cytosolic Ca^{2+} without nutrient stimulation failed to induce matrix alkalinization (10). Third, matrix acidification by nigericin neither caused mitochondrial Ca^{2+} uptake by itself nor potentiated the initial glucose-dependent mitochondrial Ca^{2+} rise. This is also consistent with our recent findings that stress the importance of the Na^{+}/Ca^{2+} exchanger as opposed to the Ca^{2+}/H^{+} transporter in the β cell. Inhibition of the Na^{+}/Ca^{2+} exchanger rescues mitochondrial dysfunction and glucose-stimulated insulin secretion in rat islets overexpressing dominant-negative PDX1 by increasing the glucose stimulated matrix Ca^{2+} signal (32).

The principal effect of matrix pH is on sustained nutrient-dependent stimulation of energy metabolism. Glucose-stimulated insulin secretion increased initially but was almost completely blocked thereafter. On the basis of its effect on metabolism-secretion coupling, matrix acidification progressively lowers mitochondrial Ca^{2+} signals as well. The described blunting of second-phase insulin secretion is therefore due to the absence of both mitochondrial activating signals. Initial metabolism-secretion coupling is much less affected, as mitochondrial Ca^{2+} reached similar maximal concentrations in the presence and absence of nigericin.

We propose that the matrix pH and mitochondrial calcium transients are complementary mechanisms regulating mitochondrial oxidative phosphorylation. Calcium signals are rapid and transient, which allows the adjustment of mitochondrial energy metabolism on a second-to-second basis. On the other hand, the matrix pH changes are slow and may modulate oxidative phosphorylation in response to long-lasting signals, such as changes in nutrient conditions.

This study demonstrates that the mitochondrial matrix pH controls the rate of oxidative phosphorylation. We propose that the low mitochondrial matrix pH observed in resting β cells is a physiological regulator that slows ATP synthesis to maintain the ATP/ADP ratio below threshold in order to prevent initiation of electrical activity. Once the matrix pH rises in response to nutrients, this inhibition is gradually lost with potentiating effects on both triggering and amplifying pathways of insulin secretion. FJ

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