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The Characterization of Mitochondrial Permeability Transition in Clonal Pancreatic β-Cells

MULTIPLE MODES AND REGULATION*

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Mitochondrial permeability transition (MPT), which contributes substantially to the regulation of normal mitochondrial metabolism, also plays a crucial role in the initiation of cell death. It is known that MPT is regulated in a tissue-specific manner. The importance of MPT in the pancreatic β-cell is heightened by the fact that mitochondrial bioenergetics serve as the main glucose-sensing regulator and energy source for insulin secretion. In the present study, using MIN6 and INS-1 β-cells, we revealed that both Ca^{2+}-phosphate- and oxidant-induced MPT is remarkably different from other tissues. Ca^{2+}-phosphate-induced transition is accompanied by a decline in mitochondrial reactive oxygen species production related to a significant potential dependence of reactive oxygen species formation in β-cell mitochondria. Hydroperoxides, which are indirect MPT co-inducers active in liver and heart mitochondria, are inefficient in β-cell mitochondria, due to the low mitochondrial ability to metabolize them. Direct cross-linking of mitochondrial thiols in pancreatic β-cells induces the opening of a low conductance ion permeability of the mitochondrial membrane instead of the full scale MPT opening typical for liver mitochondria. Low conductance MPT is independent of both endogenous and exogenous Ca^{2+}, suggesting a novel type of nonclassical MPT in β-cells. It results in the conversion of electrical transmembrane potential into ΔpH instead of a decrease in total protonmotive force, thus mitochondrial respiration remains in a controlled state. Both Ca^{2+}- and oxidant-induced MPTs are phosphate-dependent and, through the "phosphate flush" (associated with stimulation of insulin secretion), are expected to participate in the regulation in β-cell glucose-sensing and secretory activity.

Mitochondrial permeability transition (MPT)[1] is a permeability increase of the inner mitochondrial membrane to solutes of molecular mass up to ~1500 Da, which is caused by an opening of specific nonselective proteinaceous pores in the inner mitochondrial membrane (1, 2). Increased permeability of the inner membrane is initiated by an increased level of intramitochondrial Ca^{2+} and is regulated by multiple effectors, including inorganic phosphate, the redox state of pyridine nucleotides and thiols (oxidative stress), membrane potential, cyclosporin A, and other factors (3). MPT pores are thought to have at least two open conformations and a variable degree of reversibility. Most importantly, MPT in its different forms contributes to both normal cell physiology (regulation of oxidative phosphorylation and Ca^{2+} metabolism) and to regulatory processes leading to apoptosis (2–4). Specific features of MPT show significant tissue-specific variability (5–8). The most significant and well documented deviation from classical MPT described in liver mitochondria was observed in brain tissue (9). Brain mitochondria demonstrate high resistance to MPT opening (10), low sensitivity to cyclosporin A (5), different responses to MPT modifiers, and a distinctive composition of Ca^{2+}-phosphate complexes sequestered in the matrix (11). Mitochondria from skeletal muscle, unlike hepatocyte mitochondria, show the dependence of MPT on electron flux through respiratory complex 1 (12). Most importantly, basal endogenous MPT activity (transient pore opening), which affects Ca^{2+} signaling, oxidative phosphorylation, and ROS production, is different in different cell types, which can be attributed to the variable cellular redox states (13).

Recent studies in pancreatic β-cells demonstrate a dual role for MPT. Studies examining the role of cytokines in the pancreatic β-cell indicated, by means of mitochondrial depolarization and cytochrome *c* release, that MPT is a common effector of both apoptosis and necrosis (14, 15). Thus, MPT is implicated in β-cell death, which is a key factor in the etiology of type 1 diabetes and a potentially important contributor to some forms of type 2 diabetes (16). With respect to type 2 diabetes, inhibition of MPT opening with cyclosporin A was found to suppress glucose-induced insulin secretion, suggesting that a basal level of MPT transient opening (flickering) is important for β-cell secretory function (17).

Previous studies examining MPT in β-cells were performed using intact cell preparations, and the manipulation of the mitochondrial functional state, specifically controlling the levels of MPT effectors, is extremely difficult. A better understanding of MPT in the β-cell requires a more detailed investigation at the mitochondrial level. Permeabilized clonal pancreatic β-cells allow for direct experimental access to mitochondria within the cell and, therefore, provide an appropriate

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[1] The abbreviations used are: MPT, mitochondrial permeability transition; ROS, reactive oxygen species; DCF, dichlorofluorescein; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; BCECF, 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein; PhArs, phenylarsine oxide; NEM, *N*-ethylmaleimide.

system for detailed investigation of mitochondrial bioenergetics under conditions inducing MPT. The pancreatic mouse β-cell line, MIN6, was the primary model used for this study as it has been shown to retain insulin-secretory responses to glucose and other secretagogues and has been used extensively in studies of β-cell metabolism (18–20). Key findings were verified by using another widely used rat β-cell line, INS-1 (21).

We demonstrate that mitochondria from both MIN6 and INS-1 cells exhibited novel MPT characteristics as follows: (i) Ca^{2+}-phosphate-induced MPT is associated with reduced (instead of enhanced) mitochondrial ROS formation; (ii) initiation of MPT with hydroperoxides does not happen in intact β-cell mitochondria but requires exogenous glutathione peroxidase mimetic activity; and (iii) nonclassical MPT, caused by thiol cross-linking, is entirely independent of mitochondrial Ca^{2+} and causes the interconversion of electrical and chemical components of the mitochondrial protonmotive force. The role of inorganic phosphate (P_i) in MPT in the β-cell is of particular interest, as the stimulation of insulin secretion is associated with a significant drop in the P_i level ("phosphate flush"). Here we demonstrate that inorganic phosphate in the physiological range enhances the effect of Ca^{2+} on the MPT, while suppressing the effects of thiol oxidants. This supports a potential link between insulin-secretory activity and Ca^{2+}- and oxidant-induced MPT in the β-cell.

EXPERIMENTAL PROCEDURES

Reagents—Clonal pancreatic β-cells MIN6 (a gift from Dr. S. Seino, Chiba University, Japan) and INS-1 (a gift from Dr. C. Wollheim, University Medical Center, Geneva, Switzerland, passage number 68) were used in this study. Ca-Green 5N and Amplex Red were purchased from Molecular Probes (Eugene, OR), and all other chemicals were obtained from Sigma.

Growth and Permeabilization of Cells—MIN6 cells were cultured in Dulbecco's modified Eagle's medium containing 25 mM glucose and supplemented with 10% fetal bovine serum, 1 mM pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin. For INS-1 cells RPMI 1640 medium supplemented with 10% fetal bovine serum, 10 mM HEPES, 2 mM glutamine, 1 mM pyruvate, 0.05 mM β-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin was used. After 4–6 days of growth, with daily medium change, trypsinized cells were washed in Ca^{2+}-free Krebs-Ringer buffer (KRB buffer, 120 mM NaCl, 1.0 mM $MgCl_2$, 24 mM $NaHCO_3$, and 10 mM HEPES, pH 7.3) and permeabilized essentially as described by Civelek *et al.* (22). Briefly, ~2×10^7 cells were suspended in 0.7 ml of KRB buffer containing 80 μg/ml saponin. After incubation at room temperature for 5 min, the cells were centrifuged (735 × *g* for 3 min) at 4 °C and washed in cold KRB buffer. At this point more than a half of the total cellular protein and more than 80% of soluble malate dehydrogenase was found in the supernatant. Finally, the permeabilized cells were suspended in cold 0.25 M sucrose containing 10 mM HEPES, pH 7.3, and stored at 0–4 °C until required. All experiments were performed in the MIN6 line origin, and the main findings were validated by using INS-1 β-cells.

Respiration Measurements—Mitochondrial O_2 consumption was measured using a Clark-type electrode coupled to an Oxygraph unit (Hansatech, Pentney, UK). Permeabilized cells were suspended at a concentration of 0.6–0.9 mg of protein/ml in incubation medium containing 0.25 M sucrose, 10 mM HEPES, 1 mM $MgCl_2$, 20 μM EGTA, 0.1% bovine serum albumin, varying concentrations of P_i, pH 7.3 (adjusted with KOH). Glycerol 3-phosphate (7.5 mM), 10 mM succinate, 5/5 mM glutamate/malate were used as respiratory substrates. Oxygen kinetic traces were treated as described by Estabrook (23), and respiration rates were converted into molar oxygen units using O_2 solubility in sucrose medium, as reported by Reynafarje *et al.* (24).

Mitochondrial Membrane Potential Monitoring—Mitchondrial membrane potential was monitored by observing safranin O fluorescence (25, 26) in suspensions of permeabilized cells. Measurements were performed using a FluoroCount plate reader (Packard Instrument Co.) at excitation/emission wavelengths of 530/590 nm. A decrease in fluorescence corresponded to an increase in mitochondrial membrane potential. Incubation medium for permeabilized cells was essentially identical to that used for respiratory assays but was further supplemented with 2.5 μM safranin.

NAD(P)H Assay and Intramitochondrial pH Measurement—The extent of mitochondrial NAD(P)H reduction in permeabilized cells was estimated by fluorescence at excitation/emission wavelengths of 360/460 nm according to Ref. 27. To estimate dynamics of intramitochondrial pH, permeabilized cells were loaded with BCECF-AM essentially as described previously (28). Epifluorescent imaging of individual BCECF-loaded cells was performed using an Olympus IX70 inverted epifluorescence microscope with a 40× oil immersion objective, in combination with an Ultrapix camera and a PC computer with Merlin imaging software (LSR Inc., UK). BCECF fluorescence from the cell suspension was monitored with a FluoroCount plate reader at excitation/emission wavelengths of 490/530 nm.

Fluorometric Determination of Reactive Oxygen Species and Cytochrome c Assay—ROS production was assayed as hydrogen peroxide formation by monitoring fluorescein or Amplex Red oxidation in the presence of horseradish peroxidase (29, 30). Appearance of dichlorofluorescein (DCF) from nonfluorescent reduced form was monitored at excitation/emission wavelengths 485/530 nm. DCF was obtained from the stable compound DCF diacetate by alkaline hydrolysis (31). For Amplex Red measurement, wavelengths of 550/590 nm were used. The high concentrations (1.55 units/ml) and the low K_m value of peroxidase helps to circumvent interference from endogenous hydrogen peroxide-metabolizing enzymes (32). Cytochrome *c* release from the intermembrane mitochondrial space was estimated by assaying it in the incubation medium, using a sandwich enzyme immunoassay kit from R & D Systems (Minneapolis, MN) (30).

Insulin Secretion Assay—MIN6 cells cultured in 12-well plates were washed and preincubated for two sequential 30-min periods in a modified glucose-free Krebs-Ringer buffer (KRB buffer, 115 mM NaCl, 5.0 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 24 mM $NaHCO_3$, and 10 mM HEPES, pH 7.3) with 0.1% bovine serum albumin, followed by incubation for 1 h in the same buffer containing 0 or 16.7 mM glucose in the absence or presence of 5 μM cyclosporin A. Insulin secretion in response to glucose was quantified using a radioimmunoassay (Linco Research, St. Charles, MO) according to the manufacturer's instructions.

Statistical Analysis—Data were analyzed using an unpaired two-tailed Student's *t* test. Statistical significance was assumed at $p < 0.05$.

RESULTS

Calcium Phosphate-induced MPT and Mitochondrial ROS Production—Because experiments in permeabilized β-cells were supplemented with respiratory substrate but not ATP, only mitochondrial Ca^{2+} stores were available under these experimental conditions. This was verified by using inhibitors of mitochondrial and endoplasmic reticulum Ca^{2+} uptake as shown in Fig. 1. Glycerol 3-phosphate was used as the main respiratory substrate for two reasons. First, because of the high glycerol-3-phosphate dehydrogenase activity in pancreatic β-cells (27), this substrate exhibits the highest respiratory control ratio, and as such, it is possible to observe the full range of mitochondrial functional states. Second, glycerol 3-phosphate is a physiologically important mitochondrial substrate in β-cells where the glycerophosphate shuttle ensures coupling between glycolysis and mitochondrial oxidation (27) and takes part in the activation of insulin secretion (33). MIN6 cells exhibit high activity of glycerol-3-phosphate dehydrogenase similar to that in primary β-cells (34). Glycerol-3-phosphate dehydrogenase is activated by divalent cations, including the main MPT-inducer Ca^{2+} (35). Therefore, to ensure independence of glycerol-3-phosphate dehydrogenase activity on the experimental variation in Ca^{2+} concentration, the medium contained a high concentration of glycerol 3-phosphate (7.5 mM) and a physiological level of magnesium (1 mM). The presence of magnesium also maintained optimal mitochondrial coupling and did not qualitatively change mitochondrial responses to MPT inducers.

The suitability of permeabilized β-cells for studying MPT is demonstrated by Fig. 2*A*, showing gradual acceleration of respiration, decreased membrane potential, and exhaustion of Ca^{2+} loading capacity of MIN6 cell mitochondria upon titration with Ca^{2+}, which are typical characteristics of MPT. At moderate Ca^{2+} loads (<60–80 μM), MPT was reversible as indicated by the restoration of membrane potential and state 4

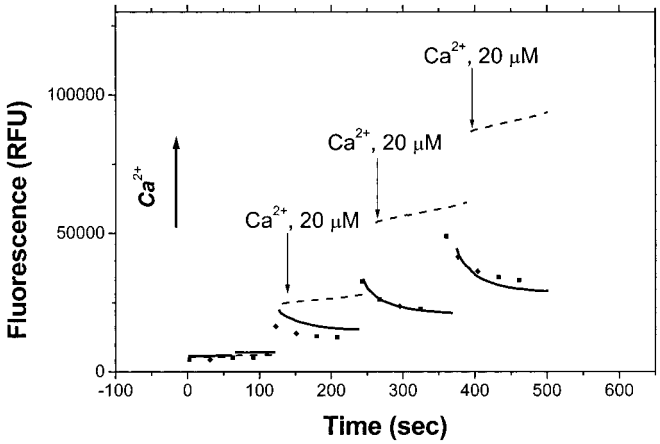

FIG. 1. **Mitochondrial character of Ca^{2+} uptake in suspension of permeabilized MIN6 cells.** Permeabilized cells were suspended in the medium for respiration assays (0.8 mg of protein/ml) and titrated with exogenous $CaCl_2$ (indicated by *thin arrows*). Calcium concentration in the medium was monitored using cellular Ca^{2+} Green-5N fluorescence. The negligible contribution of the endoplasmic reticulum on cellular Ca^{2+} uptake under the present experimental conditions is demonstrated by comparison of mitochondrial and total Ca^{2+} uptake. Mitochondrial Ca^{2+} uptake (*solid trace*) was monitored in the presence of respiratory substrate (7.5 mM glycerol 3-phosphate) and endoplasmic reticulum stores inhibitor (0.5 mM vanadate). Total Ca^{2+} uptake (*dotted trace*) was determined by incubation with the respiratory substrate alone. In control titration (*dashed trace*) both mitochondrial and endoplasmic reticulum stores were inhibited by 1 μM antimycin A and 0.5 mM vanadate, respectively. Data shown are representative of a typical experiment ($n = 3$).

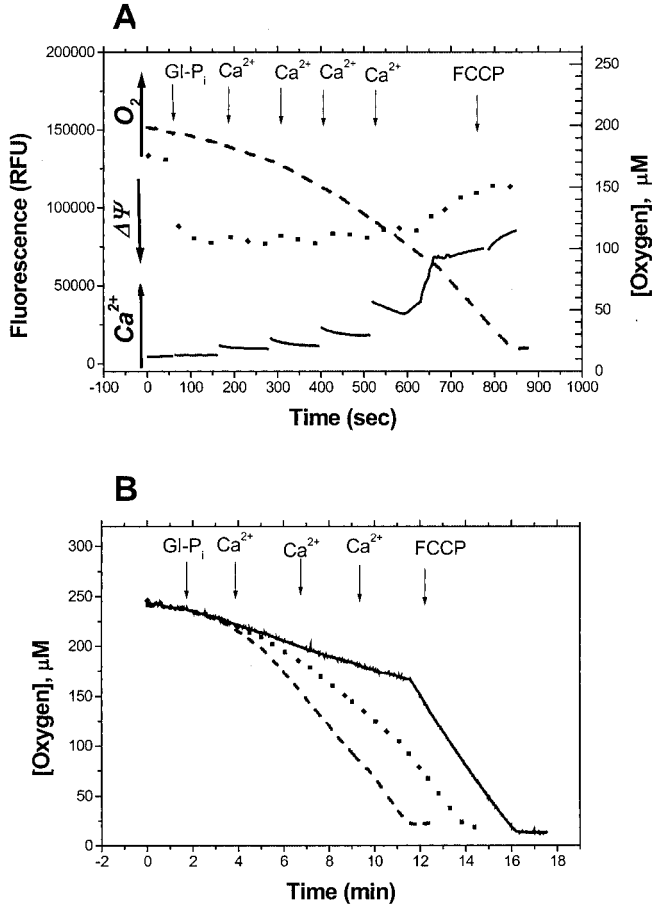

FIG. 2. **Effect of Ca^{2+} on mitochondrial function (respiration, membrane potential, and Ca^{2+}uptake) in MIN6 cells (*A*) and its dependence on P_i (*B*).** *A*, permeabilized cells were suspended in the medium used for the respiration assay (0.75 mg of protein/ml, see "Experimental Procedures") containing 2.5 mM P_i. Ca^{2+} uptake was monitored by Ca^{2+} Green-5N fluorescence (*solid trace*), membrane potential by safranin fluorescence (*dotted trace*), and mitochondrial respiration using a Clark-type electrode (*dashed trace*). Measurements were performed separately but using the same cell suspensions. Additions indicated by *thin arrows* are as follows: 7.5 mM glycerol 3-phosphate (*Gl-P*), 20 μM $CaCl_2$ (*Ca^{2+}*), and 5 μM uncoupler FCCP. Data shown are representative of a typical experiment ($n = 5$). *B*, measurements were performed under conditions shown in *A* at the following P_i concentrations: 1 mM (*solid trace*), 2.5 mM (*dotted trace*) and 5 mM (*dashed trace*). Additions indicated by *thin arrows* are as follows: 7.5 mM glycerol 3-phosphate (*Gl-P*), 20 μM $CaCl_2$ (*Ca^{2+}*), and 5 μM FCCP. Data shown are representative of a typical experiment ($n = 4$).

respiration rate upon addition of 1 μM cyclosporin A or 1 mM EGTA (data not shown). Ca^{2+} exerted these effects in the presence of 2.5 mM P_i (Fig. 2*A*), which was the first MPT co-inducer described (3). Because of the fundamental importance of P_i in MPT regulation, it is of interest that initiation and cessation of insulin secretion in β-cells is accompanied by the decrease and restoration, respectively, of the cellular P_i concentration (36–40). This so-called phosphate flush amounts to 40–50% of the initial cytosolic P_i level (2–4 mM) (38, 39). Therefore, we investigated the concentration range in which P_i regulates MPT in β-cell mitochondria in greater detail. Sustained activation of mitochondrial respiration with Ca^{2+}, reflecting MPT opening at the different P_i levels, is shown in Fig. 2*B*. Here MPT-stimulated mitochondrial oxygen consumption in β-cells is highly sensitive to P_i concentrations in the 0.5–5 mM range, suggesting that P_i-dependent MPT provides an additional link between insulin-secreting activity and mitochondrial functional state in β-cells.

Increased mitochondrial production of reactive oxygen species (ROS) is considered an essential step in the mechanism of Ca^{2+}-phosphate-induced MPT. This was deduced from the stimulation of ROS generation in the liver mitochondria respiratory chain by Ca^{2+} and P_i (42–44). The mitochondrial production of ROS could be of even greater importance in β-cells, which contain only low levels of antioxidant enzymes (45–47). Consequently, we monitored the production of H_2O_2 (estimated by Amplex Red fluorescence) which accompanied Ca^{2+}-phosphate-stimulated MPT (estimated by stimulation of mitochondrial respiration). We found that Ca^{2+} in the presence of P_i causes MPT opening (Fig. 3*B*) but inhibited, rather than stimulated, H_2O_2 production (Fig. 3*A*). Similar results were observed when ROS production was monitored with another ROS-sensitive probe (DCF) or when the order of the addition of Ca^{2+} and phosphate were reversed (data not shown).

Thus the relationship between Ca^{2+} load and mitochondrial ROS production in MIN6 β-cells is distinct from that seen in liver (42, 43) but resembles the inhibitory effect of Ca^{2+} on ROS generation reported in brain mitochondria (30). This duality of function can arise because of the variety of ways mitochondrial ROS can be induced. Mitochondrial formation of ROS results from a highly reduced state of the respiratory chain, caused by either hyperpolarization of mitochondrial membrane potential (typically complex I) or by substantial inhibition of respiratory flux (complex III) (48–51). It is thought that the most physiologically relevant mitochondrial ROS production is from complex I, in particular driven by reverse electron transfer (51). This pathway is very sensitive to membrane potential and, hence, Ca^{2+}-induced mitochondrial depolarization. Therefore, we considered if this mechanism applies to our experimental system. The ability of the respiratory substrate glycerol 3-phosphate to support reverse electron transfer is not well documented. It has been observed in insect flight muscle mitochondria (52, 53) but could not be detected in mitochondria from mouse brain and kidney (54). This discrepancy was attributed to the tissue-specific difference between the electron transfer

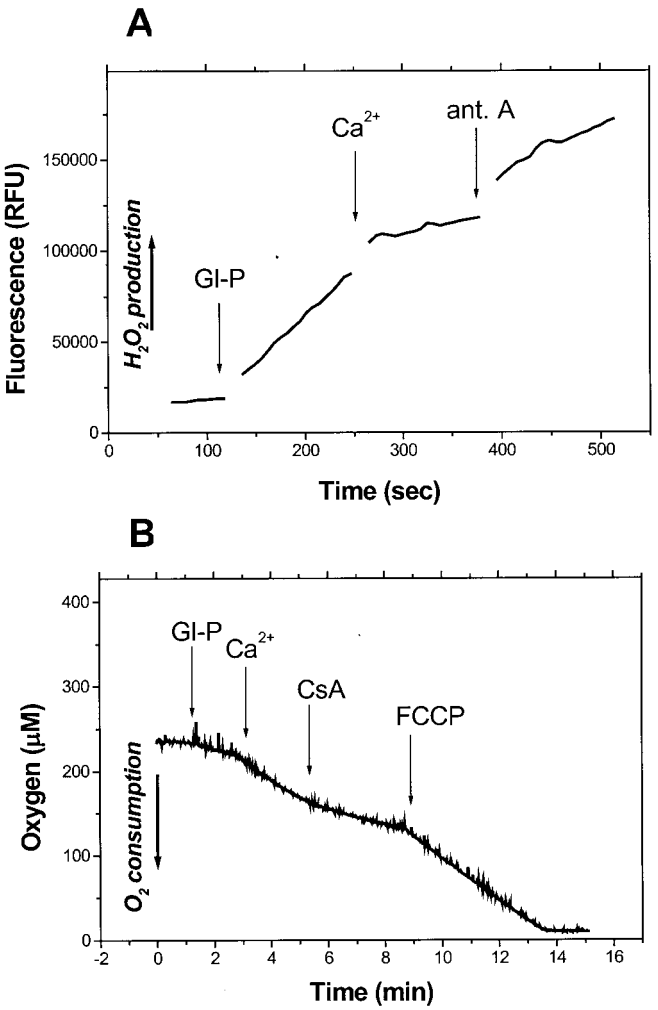

FIG. 3. **Effect of Ca^{2+} load, triggering MPT in MIN6 cells mitochondria, on mitochondrial ROS production.** Permeabilized cells were suspended in the medium for respiration assay (0.75 mg of protein/ml) containing 2.5 mM P_i and supplemented with the H_2O_2-detecting system: 1 μM Amplex Red and 0.11 μM horseradish peroxidase. Fluorescent measurement of H_2O_2 production (*A*) and polarographic measurement of respiration (*B*), demonstrating MPT onset, were performed in parallel using identical samples. Additions indicated by *thin arrows* are as follows: 7.5 mM glycerol 3-phosphate (*Gl-P*), 50 μM $CaCl_2$ (*Ca^{2+}*),1 μM antimycin A (*ant.A*), 1 μM cyclosporin A (*CsA*), and 5 μM FCCP. Data shown are representative of three independent experiments.

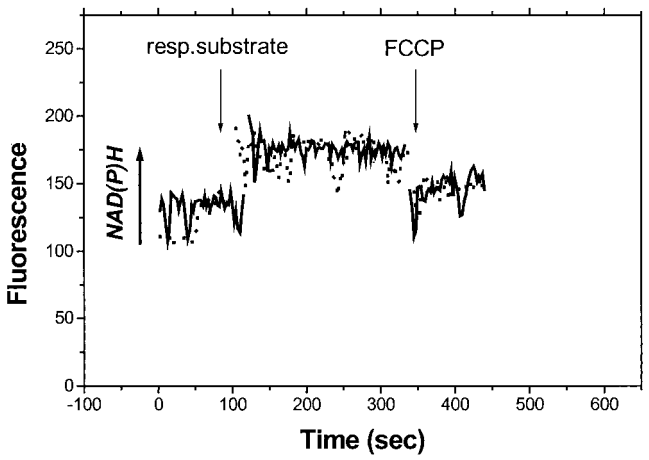

FIG. 4. **Reverse electron transport in MIN6 cells mitochondria monitored by mitochondrial NAD(P)H fluorescence.** Permeabilized cells were suspended in the medium for respiration assay (3.2 mg of protein/ml), and NAD(P)H reduction was monitored by autofluorescence at excitation/emission wavelengths of 360/460 nm fluorescence. The *thin arrow* marked "*resp. substrate*" shows initiation of respiration with 10 mM succinate (*solid trace*) or 7.5 mM glycerol 3-phosphate (*dotted trace*). The *thin arrow* marked *FCCP* indicates the addition of 6.5 μM FCCP. Data shown are representative of three independent experiments.

mechanisms of succinate and glycerol-3-phosphate dehydrogenases (54, 55). In permeabilized β-cells we found that succinate (classical substrate for reverse electron transfer) and glycerol 3-phosphate cause a similar increase of the NAD(P)H fluorescence, which is negated by the uncoupler FCCP (Fig. 4). This provides evidence for reverse electron transport from glycerol 3-phosphate to complex I, which can support the generation of ROS at this site. Indeed, glycerol 3-phosphate-dependent ROS production is inhibited by rotenone (inhibitor of complex I and reverse electron transfer) to 35 ± 3% (n = 3) of the initial rate (data not shown). This mechanism of ROS formation explains the inhibitory rather than stimulatory action of Ca^{2+} and P_i on mitochondrial oxygen radicals in MIN6 cells by Ca^{2+}-induced mitochondrial depolarization. In addition, the direct inhibitory action of Ca^{2+} on the mitochondrial H_2O_2-producing site was reported recently (56). The effect of Ca^{2+} and P_i on ROS production, supported by NAD-linked respiratory substrates (glutamate/malate), was also investigated. They were shown not to stimulate mitochondrial H_2O_2 production (data not shown).

MPT in the Presence of Hydroperoxides—In liver and heart mitochondria, hydroperoxides and other oxidants serve as potent co-inducers of MPT initiated by Ca^{2+} (1, 3). To test for the occurrence of Ca^{2+} prooxidant-induced MPT in MIN6 cells, we used *tert*-butyl and cumene hydroperoxides as models for naturally produced peroxides. Neither compound had a significant effect on membrane potential (Fig. 5), respiration, or Ca^{2+} retention in mitochondria nor did they affect Ca^{2+}-phosphate-induced mitochondrial permeabilization (data not shown). We attribute this to the fact that the effect of peroxides on MPT is mediated by the glutathione peroxidase/glutathione reductase system, which results in glutathione and NAD(P)H oxidation, an oxidative shift in the mitochondrial redox state and finally oxidation of critical thiol groups governing MPT opening (4, 57). β-Cells are known to contain very low levels of antioxidant enzymes, including glutathione peroxidase (47, 58) that may prevent mitochondrial permeabilization caused by hydroperoxides through glutathione oxidation. Indeed, mitochondrial NAD(P)H is readily oxidized by *t*-butyl hydroperoxide (0.1–0.8 mM) in hepatocytes, liver mitochondria (59, 60), and permeabilized clonal neuronal cells (57). However, in permeabilized β-cells mitochondrial NAD(P)H was not affected by *t*-butyl hydroperoxide up to a concentration of 2 mM (data not shown). The requirement of glutathione peroxidase for hydroperoxide-induced mitochondrial damage in β-cells is confirmed by the effect of the glutathione peroxidase mimetic ebselen. β-Cell mitochondria that are Ca^{2+}-loaded and treated with *t*-butyl hydroperoxide react to 10 μM ebselen with significant depolarization (Fig. 5). The protective effect of 2 mM reduced glutathione confirms that ebselen operates in a glutathione peroxidase mimic way and substitutes for low endogenous glutathione peroxidase in the initiation of mitochondrial damage.

To investigate further the effects of hydroperoxides on β-cell mitochondrial performance, we measured the respiratory control ratio (uncoupled respiration rate/state 4 respiration rate) in permeabilized MIN6 cells preincubated with *t*-butyl hydroperoxide. In contrast to liver (61), β-cell mitochondria did not show a significant decrease in respiratory control after a 10-min preincubation with 2 mM *t*-butyl hydroperoxide (respiratory control ratio was 4.54 ± 0.62 and 4.26 ± 0.26 in control

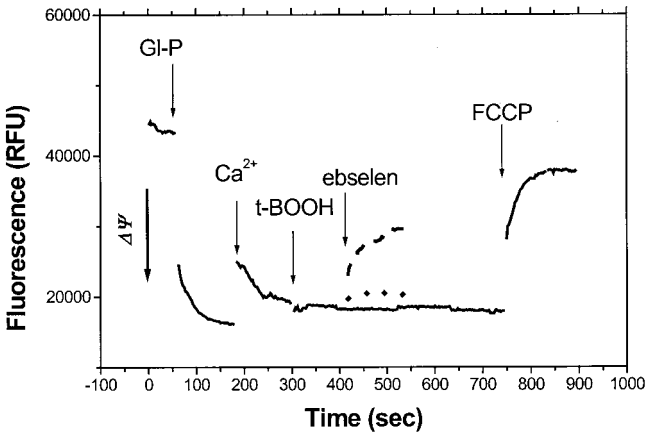

FIG. 5. **Hydroperoxide-induced mitochondrial depolarization in MIN6 cells requires presence of the glutathione peroxidase mimetic ebselen.** Permeabilized cells were suspended in the medium for respiration assay (0.75 mg of protein/ml) containing 2.5 μM safranin. *Solid trace* shows stable membrane potential in mitochondria treated with *t*-butyl hydroperoxide; *dashed trace* represents mitochondrial depolarization induced by addition of 10 μM ebselen, which was largely eliminated in the presence of 2 mM reduced glutathione (*dotted trace*). Additions indicated by *thin arrows* are as follows: 7.5 mM glycerol 3-phosphate (*Gl-P*), 20 μM $CaCl_2$ (Ca^{2+}), 0.2 mM *t*-butyl hydroperoxide (*t-BOOH*), 10 μM ebselen, and 5.6 μM FCCP. Data shown are representative of a typical experiment ($n = 3$).

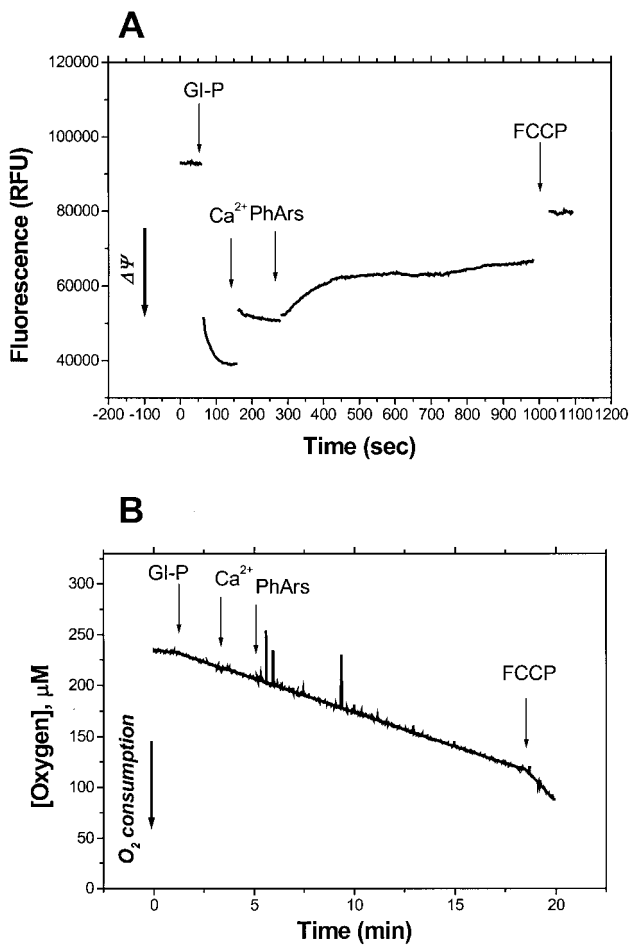

FIG. 6. **Thiol cross-linker PhArs causes mitochondrial depolarization (*A*) on retention of controlled respiration (*B*) in MIN6 cells.** Permeabilized cells were suspended in the medium for respiration assay (0.8 mg of protein/ml) to make two comparable assays to which 2.5 μM safranin was added. Additions indicated by *thin arrows* are as follows: 7.5 mM glycerol 3-phosphate (*Gl-P*), 20 μM $CaCl_2$ (Ca^{2+}), 30 μM phenylarsine oxide (*PhArs*), and 5.6 μM FCCP. Data shown are representative of a typical experiment ($n = 5$).

and hydroperoxide-treated cells, respectively, $n = 3$, $p > 0.05$). Apparent mitochondrial resistance to *t*-butyl hydroperoxide distinguishes the β-cell from other cell types and appears to contradict the known sensitivity of β-cells to oxidative stress (62). We speculated that the effect of hydroperoxides on β-cell mitochondria could be mediated by reactions of hydroperoxides in the cytosol. Hence we took NAD(P)H oxidation as an early sign of mitochondrial oxidative injury (59) and compared its occurrence in response to *t*-butyl hydroperoxide in two systems. The first was permeabilized MIN6 cells devoid of the majority of cytosolic components. The second was obtained by direct cell permeabilization in the assay mixture so that it retained all cytosolic constituents. *t*-Butyl hydroperoxide (0.2 mM) did not affect NAD(P)H levels in the first system but caused significant NAD(P)H oxidation in cellular preparations containing cytosol (data not shown).

MPT Induced by Thiol Cross-linking Agents—Unlike hydroperoxides, thiol cross-linkers induce MPT by a direct interaction with critical mitochondrial thiols (3, 4). In this study the most widely used thiol cross-linker, phenylarsine oxide (PhArs), added to β-cells loaded with 20 μM Ca^{2+} caused mitochondrial depolarization (Fig. 6*A*). However, in β-cell mitochondria, distinct from liver organelles (63), this was not accompanied by an acceleration in respiration (Fig. 6*B*). Furthermore, in contrast to liver mitochondria (64), PhArs-induced depolarization of β-cell mitochondria was insensitive to cyclosporin A concentrations which were able to prevent or revert Ca^{2+}-phosphate-induced mitochondrial permeabilization in β-cells, as well as to bongkrekic acid, which binds to the adenine nucleotide translocase and prevents Ca^{2+}-induced MPT (data not shown) (3). In liver mitochondria, PhArs, unlike other MPT inducers, is able to induce MPT in the absence of Ca^{2+}, but this effect is strongly stimulated by the addition of Ca^{2+} (64). In β-cells PhArs-induced mitochondrial depolarization does not depend on exogenous Ca^{2+}; moreover, it is not affected by EGTA (2 mM) and Br-A23187 (20 μM) which exhaust endogenous Ca^{2+} (data not shown).

The next distinctive feature of PhArs-induced depolarization in β-cell mitochondria was observed in experiments with *N*-ethylmaleimide (NEM), which in low concentration protects critical thiol groups controlling classical MPT from oxidation (cross-linking) (2–4). We found that in β-cells 20 μM NEM prevents Ca^{2+}-induced MPT but has no effect on the PhArs- or diamide-induced depolarization (data not shown). Another agent protecting against classical MPT, monobromobimane (2), even caused some acceleration of PhArs-induced mitochondrial depolarization (data not shown). Similar features were observed upon permeability transition induced by the addition of the hydrophilic thiol-oxidizing agent diamide to β-cells (data not shown). Altogether this suggests that in mitochondria from clonal β-cells, permeabilization induced by Ca^{2+}-phosphate and thiol oxidants proceeds through significantly different mechanisms.

Thiol Oxidant-induced MPT and Ion Permeability in β-Cell Mitochondria—Parallel monitoring of mitochondrial respiration and membrane potential in β-cells reveals an important feature in MPT induced by oxidants distinctive from Ca^{2+}-phosphate-induced MPT. This is the preservation of a low state 4 respiration rate accompanied by clear depolarization upon application of thiol-oxidizing agents (PhArs or diamide) (Fig. 6). This observation discriminates β-cell mitochondria from the liver organelles that respond to similar PhArs concentrations with a significant acceleration in mitochondrial respiration (63). Moreover, this observation is opposing to the typical pattern of classical uncoupling, where a significant initial increase

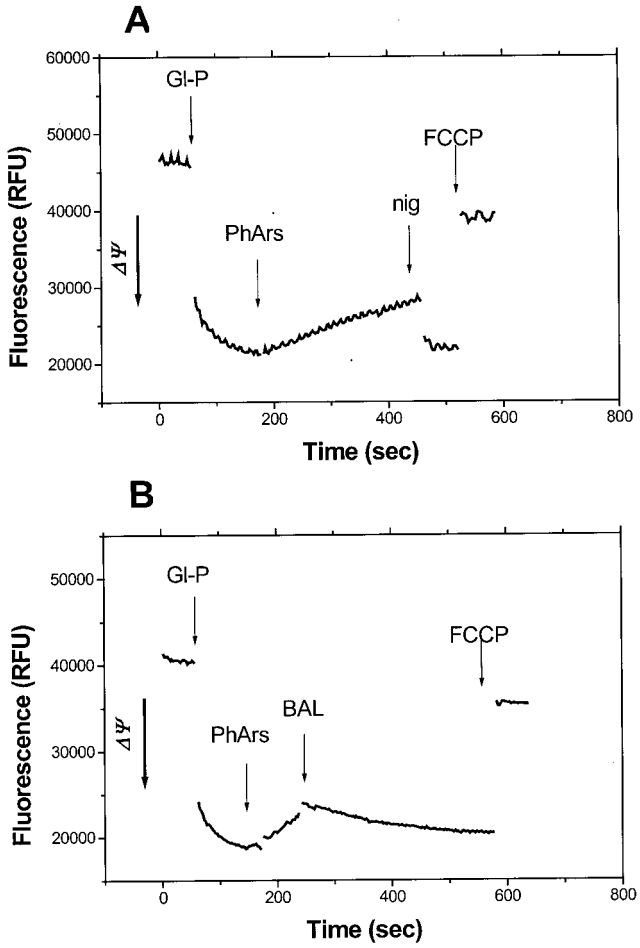

FIG. 7. **Reversibility of mitochondrial depolarization caused by PhArs upon application of nigericin (*A*) and 2,3-dimercaptopropanol (*B*).** Permeabilized cells were suspended in the medium for respiration assay (0.75 mg of protein/ml) containing 2.5 μM safranin. Additions indicated by *thin arrows* are as follows: 7.5 mM glycerol 3-phosphate (*Gl-P*), 30 μM phenylarsine oxide (*PhArs*), 2 μM nigericin (*nig*), and 30 μM 2,3-dimercaptopropanol (British antiluisite, *BAL*). Data shown are representative of four independent experiments.

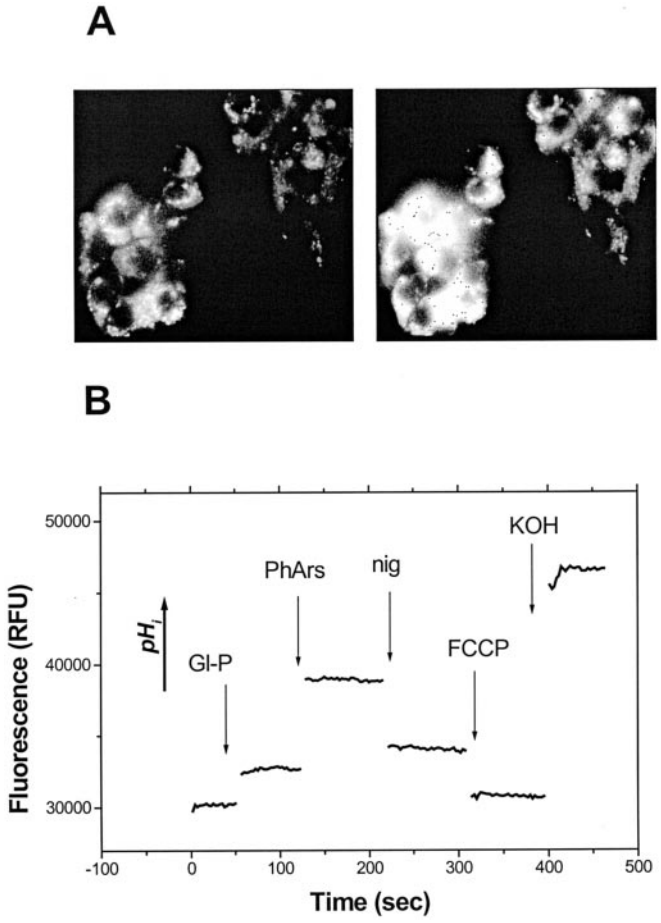

FIG. 8. **Dynamics of intramitochondrial pH in permeabilized MIN6 cells.** *A*, fluorescent images of BCECF-loaded cells before (*left*) and after (*right*) initiation of respiration by addition of 7.5 mM glycerol 3-phosphate demonstrate a basic shift of intramitochondrial pH resulting from respiration. *B*, kinetics of fluorescence in suspension of BCECF-loaded permeabilized cells (0.6 mg of protein/ml) shows alkalinization of mitochondrial matrix (increase of ΔpH) in response to PhArs and its reversal by nigericin. *Thin arrows* indicate addition of 7.5 mM glycerol 3-phosphate (*Gl-P*), 30 μM phenylarsine oxide (*PhArs*), 2 μM nigericin (*nig*), and 5.6 μM FCCP. Data shown are representative of a typical experiment ($n = 3$).

in respiration occurs with only a slight decrease in membrane potential (65, 66). This type of behavior suggests that upon thiol reagent application the total protonmotive force controlling respiration rate remains unchanged, and in fact, the apparent depolarization is the conversion of electrical potential into pH gradient. Indeed, this view is confirmed by the fact that addition of potassium/proton exchanger nigericin to β-cell mitochondria first depolarized by PhArs or diamide causes restoration of membrane potential (Fig. 7*A*). Depolarization was also suppressed by inorganic phosphate in the millimolar range (1–5 mM) in which it efficiently equilibrated pH across the mitochondrial membrane (67). For more direct evidence, permeabilized cells were loaded with the pH probe BCECF, which in this case was retained in the mitochondrial matrix. Fluorescent imaging of BCECF-loaded cells demonstrated a significant increase in fluorescence, reflecting matrix alkalinization as a result of glycerol 3-phosphate-driven respiration, as shown in Fig. 8*A*. Fluorescent monitoring of the suspension of BCECF-loaded cells shows matrix alkalinization caused by PhArs and acidification in response to nigericin (Fig. 8*B*), thus confirming PhArs-induced ΔΨ- ΔpH interconversions. Thus, the ability of the mitochondrial membrane to maintain a high potential gradient after treatment with thiol agents and nigericin suggests that it remains stringent to protons and the permeability pathway induced by these agents is the opening of the potassium-permeable pores rather than loosening of the membrane bilayer structure. In addition, mitochondrial depolarization induced by thiol cross-linking could be reverted at initial steps by the addition of a nearly stoichiometric amount of dithiol 2,3-dimercaptopropanol (Fig. 7*B*), but not monothiol 2-mercaptoethanol (data not shown). This indicates that opening and closure of PhArs-induced pores is governed by mitochondrial vicinal dithiols (68). Variation of the cationic composition of the medium suggests that this pathway is of rather broad specificity since PhArs caused mitochondrial depolarization, albeit with different rate, in potassium-, sodium-, and Tris-containing media (data not shown).

Cytochrome c Release from β-Cell Mitochondria—Extensive mitochondrial swelling following MPT opening in a high conductance mode resulted in outer membrane disruption and cytochrome *c* release, which is considered an important step in apoptotic signaling (2). A moderate increase of mitochondrial permeability induced by thiol oxidation was not expected to cause such an effect. Indeed, a comparative study of cytochrome *c* content in the cellular incubation medium after Ca^{2+}-phosphate- and PhArs-induced MPT showed that only Ca^{2+}-phosphate-induced MPT led to a statistically significant increase in cytochrome *c* release (21.5 ± 6.9% of total cyto-

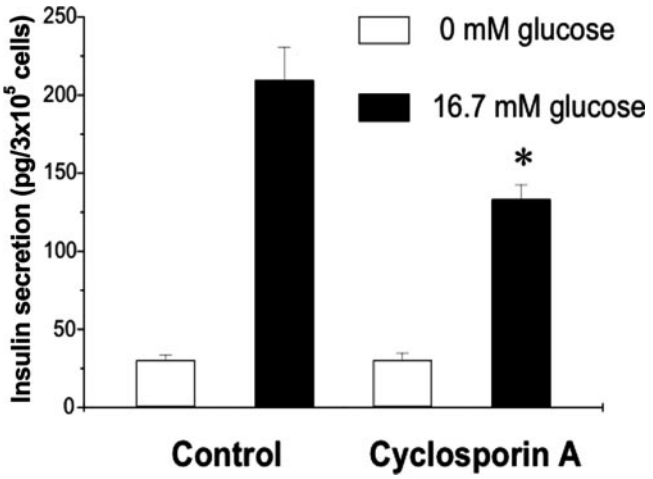

FIG. 9. **Effect of cyclosporin A on glucose-stimulated insulin secretion in MIN6 cells.** Assay was performed in standard medium (*bars* labeled *Control*) and in the medium supplemented with of 5 μM cyclosporin A (*bars* labeled *Cyclosporin A*). *, $p < 0.05$ compared with high glucose control.

chrome *c* content against 11.6 ± 5.4% in control, $n = 4$, $p < 0.05$). This confirmed the significant differences in mechanisms and consequences of the two types of mitochondrial transitions. The absence of gross changes in mitochondrial structure upon mitochondrial permeabilization induced by thiol oxidation as measured by cytochrome *c* release is in line with the aforementioned preservation of the state 4 respiration rate under these conditions. It also suggests that thiol oxidant-induced mitochondrial permeabilization to cations does not initiate cytochrome *c*-related apoptotic signaling in β-cells.

MPT and Insulin Secretion—The potential importance of basal MPT activity in β-cells for insulin secretion was deduced from the suppression of insulin secretion in mouse β-cells caused by the MPT inhibitor cyclosporin A, which was shown to be related to the mitochondrial effects of cyclosporin (17). Our data showing the inhibitor effect of cyclosporin A on glucose-stimulated insulin secretion in MIN6 cells confirms this observation (Fig. 9).

DISCUSSION

Our findings show that in the pancreatic β-cell lines MIN6 and INS-1, MPT may function in several distinct modes. The MPT features that distinguish clonal β-cells from other tissues include the following: (i) Ca^{2+}-phosphate-induced MPT is accompanied by decreased, not increased, mitochondrial ROS production; (ii) Ca^{2+}-hydroperoxide-induced MPT depends on exogenous glutathione peroxidase activity to initiate the sequence of oxidative events; (iii) nonclassical MPT caused by thiol cross-linking is independent of Ca^{2+} and leads to the conversion of $\Delta\Psi$ to ΔpH (rather than a decline in protonmotive force) and preserves respiration in a controlled state.

Ca^{2+} loading (in the presence of P_i) stimulates ROS production in liver mitochondria (43, 44), which is considered to be an intermediate step in MPT development. Mitochondrial ROS generation can proceed in two modes, one of which is stimulated by mitochondrial hyperpolarization and the other by inhibition of mitochondrial respiration (48–50). Since Ca^{2+} initially causes transient or sustained mitochondrial depolarization, it not clear how Ca^{2+} stimulates ROS production. This was explained by either massive cytochrome *c* loss resulting in severe respiratory inhibition (30) or rearrangement of the mitochondrial membrane structure in the region of superoxide-producing centers, making them more accessible to oxygen (43, 44). The main superoxide-producing centers in the mitochondrial respiratory chain are located in respiratory complexes I and III (50). Superoxide generated by complex I is considered the most physiologically relevant, since it takes place in the absence of artificial effectors (49, 51). Most importantly, we have found that complex I plays a significant role in superoxide formation in pancreatic β-cells due to reverse electron transfer from FAD-linked substrates. This type of superoxide generation strongly depends on mitochondrial membrane potential (51) and can explain superoxide suppression by the addition of Ca^{2+}. Respiration measurements upon Ca^{2+}-phosphate-induced MPT (Fig. 2) show that cytochrome *c* loss is not sufficient to inhibit respiration; thus this potential mechanism of ROS activation does not come into play here. An additional factor that is beyond the scope of our experimental model is the possible elevation of the mitochondrial level of fatty acids (arachidonic acid) caused by increased Ca^{2+} load and that is able to activate ROS production (69). Thus, because of the predominantly potential-dependent mechanism of ROS formation in β-cell mitochondria, development of Ca^{2+}-phosphate-induced MPT does not involve increased mitochondrial ROS production. However, it is likely that increased ROS stimulated by additional factors (high glucose, fatty acids, and ceramides) (62) or formed exogenously (pancreatic macrophages) (14) can significantly contribute to MPT opening in β-cells.

Tolerance of β-cell mitochondria to hydroperoxides appears atypical when compared with other cell types. The study of hepatocytes and other cells established mitochondria as a major target of hydroperoxide cytotoxicity (70). In these experiments hydroperoxides efficiently produced mitochondrial damage in intact cells, permeabilized cells, and isolated mitochondria (57, 59, 60); however, *t*-butyl hydroperoxide does not affect mitochondria in permeabilized β-cells until the system is supplemented with a glutathione peroxidase mimetic or cytosolic components. It is known that cells metabolize *t*-butyl hydroperoxide through two pathways, transforming it by glutathione peroxidase into the corresponding alcohol or cleaving it by cytochrome P-450 into alcoxyl and peroxyl radicals. The first pathway causes an oxidative shift in the cellular glutathione redox state, and the second pathway initiates and propagates lipid peroxidation (71). Products of lipid peroxidation originating from the cytosol can diffuse to mitochondria, causing oxidative damage (72). Therefore, our results suggest that in β-cells hydroperoxides initially undergo cytosolic transformation and only as a result does mitochondrial damage take place. The putative mechanism of hydroperoxide toxicity in β-cells, requiring first the extramitochondrial reactions of hydroperoxide, is essentially different from many other cell types. However, it is possible that under special circumstances a similar situation may occur in other tissues including liver. Richter and co-workers (73), investigating the effect of *t*-butyl hydroperoxide on liver mitochondria, showed that a decrease in glutathione peroxidase activity caused by a selenium-deficient diet prevented depolarization and Ca^{2+} loss caused by hydroperoxide.

The redox status of mitochondrial thiols is involved in oxidative phosphorylation and maintenance of mitochondrial membrane integrity (74). Specifically, in relation to MPT, the redox state of thiol groups in the adenine nucleotide translocase is thought to govern opening of the classical permeability transition pore (4). A typical sign of this classical transition is the protective effect of low concentrations of NEM, which alkylates these critical thiols and protects them from oxidation (4). In liver mitochondria NEM demonstrates protective efficiency against both Ca^{2+}-prooxidant- (75) and Ca^{2+}-PhArs-induced MPT (63). Therefore, the fact that in MIN6 β-cell mitochondria NEM protects against Ca^{2+}- but not PhArs-induced MPT sug-

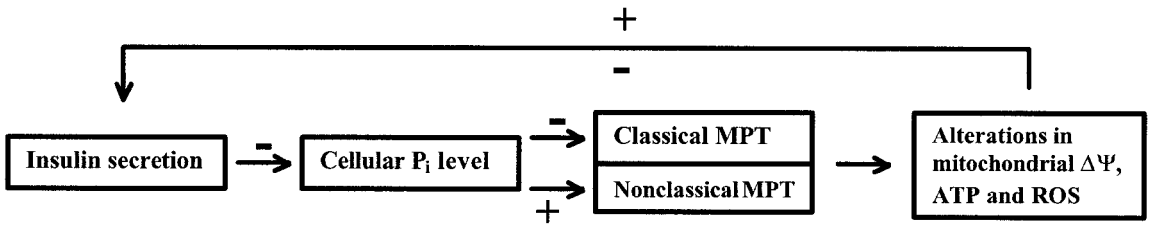

FIG. 10. **Proposed interplay of insulin secretory activity and MPTs in β-cells.** Induction of insulin secretion causes decrease in β-cell inorganic phosphate level, which suppresses classical Ca^{2+}-induced MPT and favors nonclassical MPT induced by thiol oxidation. These processes are expected to affect mitochondrial energy transformation and ROS production in a complex way determined by respiratory substrate supply, cellular energy demand, redox state, and other factors. Furthermore, these alterations in mitochondrial metabolism are able to provide feedback effects on β-cell insulin secretion.

gests that the latter is regulated by a specific set of vicinal thiols, different from ones controlling classical mitochondrial transition. MPT caused by thiol cross-linking in β-cells leaves respiration in the low controlled state and causes transformation of the electrical component of protonmotive force to ΔpH. This can stimulate ΔpH-driven mitochondrial transport of metabolites (phosphate, pyruvate, and malate) and moderate ΔΨ-driven transport (Ca^{2+} and ATP/ADP).

The effect exerted by thiol oxidants on mitochondria in clonal β-cell falls into the category of nonclassical MPTs and in some aspects resembles effects observed in the liver mitochondria exposed to Ca^{2+} and *t*-butyl hydroperoxide or diamide (76). However, nonclassical MPT in clonal β-cells differs from that described in liver in two respects. First, in MIN6 and INS-1 cells it is entirely independent of exogenous or endogenous Ca^{2+}. Second, it is suppressed by inorganic phosphate in the low millimolar range, where phosphate efficiently transforms the mitochondrial pH gradient into electrical gradient (67). Nonclassical MPT in liver is inhibited by significantly lower phosphate concentrations, which are ascribed to the binding of matrix Ca^{2+} (76). The nonclassical MPT pore is frequently considered to be either a substate of the classical MPT pore or an entirely different structure (6). We believe that MPT induced in β-cells by thiol cross-linking is more likely to be structurally different from classical MPT because of the following: (i) its insensitivity to cyclosporin A, suggesting that cyclophilin is not a component of this mechanism; (ii) the lack of a protective effect of low concentrations of NEM (protecting against classical MPT in the same cells) suggesting that this transition is regulated by the set of vicinal thiols completely different from the one regulating classical MPT; (iii) independence from Ca^{2+} and insensitivity to bongkrekic acid argue against the involvement of the adenine nucleotide translocase that has an essential requirement for Ca^{2+} for transformation into nonspecific channels (77). This kind of transition could be realized by the opening of a nonspecific cationic channel of low conductance. So far nonspecific cationic permeability was observed in liver and heart mitochondria only after Mg^{2+} exhaustion (2). Since mitochondrial ion channels often share some properties with plasma membrane channels (78), it is of interest that a nonselective redox-regulated cationic channel was found in the plasma membrane of the rat clonal insulin-secreting cell line CRI-G1 (79). As well, the existence of ion channels regulated by the redox state of thiol groups, reported in other tissues (76), suggests that this novel Ca^{2+}-independent MPT in β-cells could be a manifestation of such a channel.

The physiological role for MPT in the β-cell physiology can in part be realized through the effects of inorganic phosphate. This anion is a potent regulator of both Ca^{2+}- and oxidant-induced MPT in β-cells and is closely linked to the regulation of insulin secretion. Stimulation of insulin secretion is accompanied by a phosphate flush, which releases up to half of the β-cell phosphate content (38, 39). The normal phosphate level in β-cells is about 2–4 mM (41); thus, changes in its concentration in response to the stimulation of insulin secretion is within the range where mitochondrial susceptibility to Ca^{2+}-induced MPT is highly sensitive to phosphate (Fig. 3). The present study provides further evidence on the physiological significance of phosphate flush in β-cells. Since phosphate complexes free Ca^{2+} (65), the decrease in the cytosolic phosphate level accompanying the stimulation of insulin secretion was considered to be a contributor to the elevation of cytosolic free Ca^{2+} that triggers insulin exocytosis (37). Our data suggest an additional physiological function for this phenomenon. In addition to the stimulation of insulin exocytosis, the increase in cytosolic Ca^{2+} can raise the mitochondrial Ca^{2+} concentration, resulting in MPT opening (flickering), which compromises mitochondrial ATP synthesis in the course of insulin secretion. Therefore, phosphate flush, diminishing the cellular level of MPT co-inducer P_i, can counteract the effect of increased Ca^{2+} and keep MPT activity low during insulin secretion. Conversely, the higher level of P_i in the resting β-cell makes mitochondria more prone to Ca^{2+}-induced MPT, which may also have physiological significance. In the basal state, the β-cell tends to hyperpolarize mitochondria, which can lead to enhanced ROS production (65). Since MPT can lead to "mild uncoupling," preventing mitochondrial hyperpolarization and excessive ROS formation (13), increased levels of the MPT co-inducer P_i in the resting β-cell can protect it from oxidative stress. In contrast, mitochondrial depolarization in the β-cell caused by thiol oxidation is activated at lowered phosphate levels. Since phosphate flush reduces cytosolic P_i by ~50%, it can reach levels where the mitochondrial effects of thiol oxidants become significant. Therefore, one can expect that mitochondria in β-cells actively secreting insulin are more sensitive to oxidative stress than in resting cells. By taking into account feedback mechanisms and a variety of possible metabolic effects of different kinds of MPT, the scheme in Fig. 10 illustrates possible relationships between β-cell stimulus secretion coupling and MPT.

In summary, β-cell mitochondria, in addition to classical Ca^{2+}-induced MPT, exhibit a novel Ca^{2+}-independent type of MPT caused by thiol oxidation. These mechanisms may correlate with β-cell function in opposing ways such that the stimulation of insulin secretion makes the β-cell more sensitive to the mitochondrial effects of oxidants but more resistant to Ca^{2+}-induced mitochondrial permeabilization. In the present study the discrimination between classical and nonclassical MPT in β-cells required parallel monitoring of different mitochondrial parameters under identical conditions and therefore using clonal cell suspensions. Extension of MPT studies to the primary cells will require a single cell approach that is now being planned in our laboratory.

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