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Limited Mitochondrial Permeabilization Is an Early Manifestation of Palmitate-induced Lipotoxicity in Pancreatic β-Cells*[S]

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Involvement of the mitochondrial permeability transition (MPT) pore in early stages of lipotoxic stress in the pancreatic β-cell lines MIN6 and INS-1 was the focus of this study. Both long term (indirect) and acute (direct) effects of fatty acid (FA) application on β-cell susceptibility to Ca^{2+}-induced MPT induction were examined using both permeabilized and intact β-cells. Long term exposure to moderate (*i.e.* below cytotoxic) levels of the saturated FA palmitate sensitized β-cell mitochondria to MPT induced by Ca^{2+}. Long term exposure to palmitate was significantly a more efficient inducer of MPT than the unsaturated FA oleate, although upon acute application both caused similar MPT activation. Application of antioxidants, inhibitors of the ceramide pathway, or modifiers of membrane fluidity did not protect β-cell mitochondria from FA exposure. However, significant protection was provided by co-application of the unsaturated FA oleate in a phosphatidylinositol 3-kinase-dependent manner. Characterization of MPT pore opening in response to moderate palmitate treatment revealed the opening of a unique form of MPT in β-cells as it encompassed features of both low and high conductance MPT states. Specifically, this MPT showed solute selectivity, characteristic of a low conductance MPT; however, it affected mitochondrial respiration and membrane potential in a way typical of a high conductance MPT. Activation of the full-size/high conductance form of MPT required application of high levels of FA that reduced growth and initiated apoptosis. These findings suggest that in the β-cell, MPTs can act as both initiators of cell death and as versatile modulators of cell metabolism, depending on the mode of the MPT pore induced.

Mitochondrial permeability transition (MPT)[2] is the calcium (Ca^{2+})-dependent opening of a nonspecific pore in the mitochondrial inner membrane in response to a variety of inducers and co-inducers, including inorganic phosphate (P_i), thiol oxidants, fatty acids (FA), and others. Opening of the "full-size" or "high conductance" MPT causes equilibration across the inner membrane of ions and all solutes up to 1.5 kDa, which induces mitochondrial swelling and the release of mitochondrial proteins capable of apoptosis activation (1–5). MPT can also operate in a more "limited size" or "low conductance" mode, which allows passage of only small ions across the inner mitochondrial membrane (6, 7). Because opening of the low conductance MPT does not allow passage of large molecules, but does allow the passage of small ions, mitochondrial ion gradients and energy production are affected. As such, it is thought that the low conductance MPT functions not to induce cell death but to aid in the fine regulation of cell metabolism (6). Extensive evidence documents the high conductance MPT as a cause of generalized mitochondrial dysfunction and cell death, and an important mechanism in disease pathologies such as hepatotoxicity (8), cardiac ischemia/reperfusion (9, 10), and neuronal injury (10, 11). In contrast, low conductance MPT has received far less attention despite reports suggesting it plays an important regulatory role in cell metabolism (12–14).

The physiological and pathological significance of MPT in pancreatic β-cells is currently under investigation by several groups. According to recent studies, the induction of cell death in clonal β-cells and pancreatic islets by elevated fatty acids (FA) or other factors involves MPT opening and the release of apoptogenic factors (15–17). However, we and others suggest that the more limited forms of MPT may lead to the impairment of glucose-stimulated insulin secretion (GSIS) from β-cells, an event that likely precedes or is independent of MPT-associated apoptosis (18–21). The occurrence of both high and low conductance MPT in β-cells and their impact on β-cell viability and GSIS, respectively, suggest that MPTs may play a significant role in the initial pathogenesis of diabetes.

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[2] The abbreviations used are: MPT, mitochondrial permeability transition; GSIS, glucose-stimulated insulin secretion; FA, fatty acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; CsA, cyclosporin A; DTNB, 5,5′-dithiobis-2-nitrobenzoate; KRB, Krebs-Ringer buffer; $\Delta\Psi_m$, mitochondrial membrane potential; MDR pump, multidrug resistance pump; CsH, cyclosporin H; BSA, bovine serum albumin; PI, phosphatidylinositol; ROS, reactive oxygen species; HNE, 4-hydroxynonenal; NAC, *N*-acetylcysteine.

Chronically elevated FAs (lipotoxicity), a condition often associated with obesity, has deleterious effects on β-cell function and is considered one of the main diabetogenic mechanisms. As such, we examined the effects of saturated and unsaturated FAs on MPT in pancreatic β-cells using as our model system MIN6 and INS-1 β-cell lines. Although lipotoxicity has been extensively studied in these cell lines (17, 22–24), the function of full-size and limited size MPT induction in response to FAs has not been previously examined. In this study, we applied moderate concentrations of FA, which are known not to cause apoptosis, as a model of the early stages in the lipotoxic mechanism. This study revealed that long term (48 h) exposure of clonal pancreatic β-cells to palmitate, a saturated FA, caused subsequent activation of MPT in a dose-dependent manner. Acute application of palmitate (applied directly to permeabilized β-cells at the time of assay) revealed similar results. In contrast, the unsaturated FA oleate induced MPT upon acute application but counteracted sensitization to MPT caused by palmitate during long term exposure. The ability of oleate to exert an opposite effect on mitochondrial permeabilization reflects current controversies in the literature, where both activation (17) and protection against MPT by oleate (24, 25) were reported. Application of moderate (noncytotoxic) levels of palmitate activated a limited size (acetyl-CoA-impermeable) form of MPT, whereas activation of full-size (acetyl-CoA-permeable) MPT required exposure to higher (cytotoxic) concentrations of palmitate. Unique features of the limited size MPT in β-cells, which distinguish it from the limited size MPTs observed in other cell types, were determined. Graded activation of different MPT forms by FAs suggested involvement of MPT not only in triggering cell death but also in the metabolic regulation of β-cells.

EXPERIMENTAL PROCEDURES

Reagents and Materials—Clonal pancreatic MIN6 (a gift from Dr. S. Seino, Chiba University, passage number 40-50) and INS-1 (a gift from Dr. C. Wollheim, University Medical Center, Geneva, passage number 60-80) β-cells were used. Mouse hepatocytes were obtained as described by others (26). Ca-Green 5N and Fluo-4 were purchased from Molecular Probes (Eugene, OR); cyclosporin H was purchased from Eton Bioscience (San Diego); 4-hydroxynonenal (HNE) was obtained from Cayman Chemical (Ann Arbor, MI); and all other chemicals were obtained from Sigma. Akt phosphorylation was measured with a cell-based enzyme-linked immunosorbent assay kit (Super Array, Frederick, MD).

Growth, Treatment, and Permeabilization of β-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. INS-1 cells were cultured in 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. After 4–6 days of growth with daily medium change, cells were trypsinized and 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 previously (27). 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 half of the total cellular protein and more than 80% of soluble malate dehydrogenase were 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 for experimentation. All experiments were performed on MIN6 cells, and the main findings were validated using INS-1 β-cells.

For long term FA exposure, cells were exposed to FA in growth medium supplemented with FA bound to bovine serum albumin (BSA) (27) for 48 h prior to permeabilization and subsequent experimentation. See under "Results" for the concentrations of FA applied. For acute FA application, FAs were added directly to the assay medium containing permeabilized cells. Again, FA concentrations applied to cells are outlined under "Results."

Respiration Measurements—Mitochondrial O_2 consumption was measured using a Clark-type oxygen 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, 20 μM EGTA, 0.1% BSA, 2.5 mM KH_2PO_4, 7.5 mM glycerol 3-phosphate, pH 7.3 (adjusted with KOH). Oxygen kinetic traces were treated as described by Estabrook (28), and respiration rates were converted into molar oxygen units using O_2 solubility in sucrose medium, as reported by Reynafarje *et al.* (29).

Mitochondrial Membrane Potential (Ψ_m) and Ca^{2+} Monitoring—Mitchondrial membrane potential was monitored by observing safranin-O fluorescence (30, 31) in suspensions of permeabilized cells. Permeabilized cells were incubated in medium that was essentially identical to that used for respiratory assays (see above) but was further supplemented with 2.5 μM safranin. Measurements were performed using a Fluoro-Count 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. Extramitochondrial Ca^{2+} was monitored fluorometrically using 1 μM Ca-Green 5N at excitation/emission wavelengths of 495/530 nm.

Estimation of Permeability Transition in β-Cell Mitochondria in Situ—MPT within permeabilized cells was routinely estimated by monitoring mitochondrial membrane potential (Ψ_m), which was further verified with Ca^{2+} and respiration measurements. The mitochondrial origin of the signals was tested using the mitochondrial uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and the MPT inhibitor cyclosporin A (CsA). See the individual figure legends for concentrations of FCCP and CsA applied to cells. Mitochondria were energized by oxidation of glycerol 3-phosphate (7.5 mM), which was used as the principal respiratory substrate because glycerol-3-phosphate dehydrogenase is the most active dehydrogenase in pancreatic β-cells (32). $\Delta\Psi$ and Ca^{2+} kinetics reflect both low and high conductance mitochondrial permeabilization, and specific detection of the high conductance per-

meability transition in isolated mitochondria is commonly performed using a swelling method (4). Because this method cannot be applied to mitochondria in permeabilized β-cells, we discriminated between high and low conductance MPTs directly using a citrate synthase assay (33). In this assay opening of the large diameter pore in the inner mitochondrial membrane allows access of citrate synthase substrates (acetyl-CoA) to citrate synthase in the mitochondrial matrix. CoA-SH produced in the course of the citrate synthase reaction reduces 5,5′-dithiobis-(2-nitrobenzoate) (DTNB), which registers as a rise in absorbance at 412 nm. See Fig. 6*A* for a schematic of this assay.

Cytotoxicity Detection—Cytotoxic effect in β-cells was estimated by detecting exposure of phosphatidylserine on the plasma membrane using annexin V-fluorescein isothiocyanate according to manufacturer's (Sigma) instructions. This measurement reflects early apoptotic events and, according to recent reports (34, 35), necrotic cellular alteration. Epifluorescent imaging of cells treated with annexin V-fluorescein isothiocyanate was performed at excitation/emission wavelengths of 495/530 nm 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).

Estimation of Permeability Transition in Intact β-Cells—MPT in intact cells was estimated by monitoring MPT-related changes in intracellular Ca^{2+} and mitochondrial membrane potential ($\Delta\Psi_m$) (14, 36–38). Briefly, trypsinized cells were washed in Krebs-Ringer buffer (KRB buffer, 120 mM NaCl, 5.4 mM KCl, 10 mM HEPES, 24 mM $NaHCO_3$, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 2.8 mM glucose, pH 7.3) and suspended in the same buffer supplemented with 0.1% BSA at ~10 mg of protein/ml. For intracellular Ca^{2+} monitoring cell suspensions were loaded with 2 μM Fluo-4-AM for 30 min at room temperature, washed, suspended in the same volume of KRB/BSA buffer, and incubated for another 30 min for complete hydrolysis. For kinetic Ca^{2+} measurements, loaded cells were diluted in KRB/BSA buffer up to 0.6–0.7 mg of protein/ml and Fluo-4 fluorescence was monitored with a FluoroCount plate reader (Packard Instrument Co.) at excitation/emission wavelengths of 485/530 nm. Cellular loading of Fluo-4 was estimated by comparing the fluorescent signals from a standard solution of the free acid form of Fluo-4 and the loaded cell suspension permeabilized with 80 μg/ml saponin (39). Calibration of the fluorescent signal was performed according to a conventional procedure developed for Fluo-3 (40) using the Grynkiewicz equation $[Ca^{2+}] = K_d (F - F_{min})/(F_{max} - F)$, where the K_d employed was 345 nM (41). Essentially the same procedure was employed for the $\Delta\Psi$ measurement except the cells were loaded with 10 μM rhodamine 123 for 20 min. Cyclosporin H (CsH), which has been shown to inhibit the MDR pump (2), was used as a control for the possible contribution of MDR pump-associated fluorescent probe extrusion. Also, it has been demonstrated that anion transporters (MDR-related proteins) can also contribute to fluorescent probe extrusion. To control for this, probenecid, which has been shown to inhibit these anion transporters in β-cells (42, 43) as well as in other tissues (40, 44), was employed.

Insulin Secretion Assay and Measurement of Akt Phosphorylation—MIN6 cells cultured in 24-well plates were washed and preincubated for two sequential 30-min periods in glucose-free KRB buffer (125 mM NaCl, 5.9 mM KCl, 1.28 mM $CaCl_2$, 5.0 mM $NaHCO_3$, 25 mM HEPES, and 0.1% (w/v) bovine serum albumin), followed by incubation for 1 h in the same buffer containing 1 or 20 mM glucose in the absence or presence of 5 μM cyclosporin A or 0.15 μM FK-506. GSIS was quantified by radioimmunoassay (Linco Research, St. Charles, MO) as described previously (45).

Phosphorylation status of Akt was evaluated with a cell-based enzyme-linked immunosorbent assay kit from SuperArray (Frederick, MD). This protocol, which is based on antigen-antibody recognition, allows direct and accurate quantification of both phosphorylated and total Akt at the same time (46). The amount of phosphorylated Akt was normalized to total Akt levels and corrected for nonspecific staining by comparison with samples incubated without primary antibodies (according to the manufacturer).

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

RESULTS

Moderate Fatty Acid Treatment Does Not Induce Apoptosis in Clonal β-Cell Lines—Cells were exposed to various concentrations of palmitate, a saturated FA, and assayed for the onset of apoptosis after 48 h using the annexin V assay. In MIN6 cells, the optimal level of palmitate was found to be 0.5 mM (equilibrated with 0.5% BSA). At this concentration of palmitate exposure, growth of MIN6 cells was found to be 92 ± 12% ($p > 0.05$, $n = 4$) of control, as estimated by total cellular protein content, and no detectable apoptosis was observed (Fig. 1). In INS-1 cells, the concentration of palmitate exposure was reduced to 0.10–0.15 mM to maintain cell growth unperturbed. This is close to (47) or somewhat lower (17) than FA levels commonly used in lipotoxic studies on these cell lines. Thus, these conditions were used to model the initial steps of FA-induced β-cell deterioration without pronounced cell death.

Acute Application of FAs to β-Cells Induces a CsA-sensitive MPT upon Ca^{2+} Application—It has long been known that free FAs can directly impact mitochondrial function either through direct inhibition of respiration, respiratory uncoupling, or through the induction of MPT formation (48, 49). The impact of FAs on mitochondrial function can vary with the type of mitochondria, type of FA, length of FA exposure, and concentration of FA applied (49, 50). Therefore, we first tested the impact of acute FA application of various concentrations on mitochondrial function to determine a threshold concentration of FA that acutely induces MPT without direct respiratory inhibition or strong uncoupling. An appreciable inhibition of state 3_{FCCP} respiration (>20% decrease) was found when FA total concentration exceeded 50 μM (data not shown). Mitochondrial respiration and membrane potential in state 4 also remained stable at FA levels below 50 μM. MPT opening was then monitored by Ca^{2+}-induced mitochondrial depolarization that displayed sensitivity to CsA (a commonly used inhibitor of MPT opening). Importantly, we observed that 25 μM

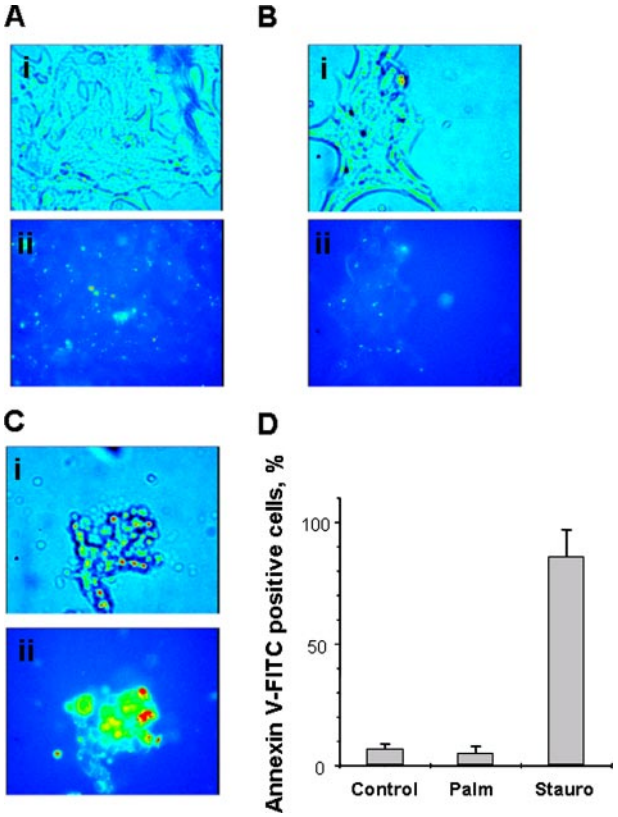

FIGURE 1. **Detection of early apoptotic events in MIN6 β-cells demonstrates an absence of cytotoxicity after long term (48 h) exposure to palmitate.** *A*, control MIN6 cells. Representative light (*panel i*) and fluorescence images (*panel ii*) of control MIN6 cells treated with annexin V to determine the presence of apoptotic cell death are shown. *B*, intact MIN6 cells were exposed to 0.5 mM palmitate (*Palm*) for 48 h. Representative light (*panel i*) and fluorescence images (*panel ii*) of palmitate-exposed MIN6 cells treated with annexin V are shown. *C*, MIN6 cells treated with the well known inducer of apoptosis, staurosporine (*Stauro*) (2 μM). Representative light (*panel i*) and fluorescent (*panel ii*) images are shown. *D*, quantification of annexin V-fluorescein isothiocyanate positive cells. Images and data shown are representative of three independent experiments.

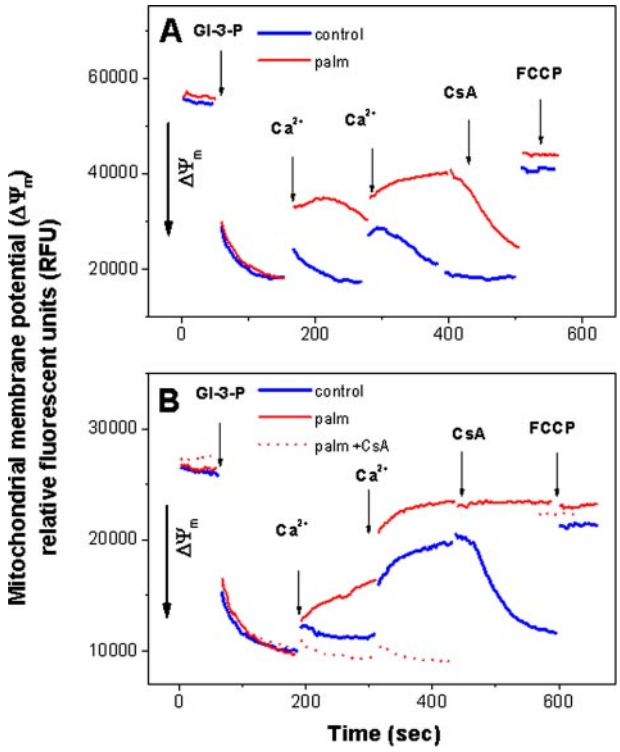

FIGURE 2. **Long term exposure of clonal β-cells to palmitate predisposes mitochondria to subsequent Ca^{2+}-induced depolarization indicative of MPT.** *A*, intact MIN6 cells were exposed to 0.5 mM palmitate (*palm*)/BSA (BSA only control), permeabilized with saponin, and suspended in incubation medium at 0.75 mg of protein/ml (see "Experimental Procedures"). Mitochondrial membrane potential was monitored by safranin-O fluorescence (downward deflection reflects an increase in potential). Additions indicated by *arrows* are as follows: 7.5 mM glycerol 3-phosphate (*Gl-3-P*), 50 μM $CaCl_2$ (*Ca^{2+}*), 1 μM CsA, and 5 μM FCCP (a mitochondrial respiratory uncoupler). Data shown are representative of a typical experiment ($n = 5$). *B*, intact INS-1 cells were exposed to 0.1 mM palmitate for 48 h, permeabilized with saponin, and analyzed under the conditions described in *A*, except that Ca^{2+} application in this case was in increments of 25 μM. The *red dashed trace* represents palmitate-exposed cells treated with cyclosporin A (1 μM) at the beginning of assay to demonstrate the classical nature of MPT induction in INS-1 cells. Data shown are representative of three independent experiments.

palmitate and oleate significantly stimulated MPT pore opening at this low concentration (shown in Fig. 4*A*). Similar stimulation was reported in liver and heart mitochondria (51, 52); however, in these studies MPT-inducing unbound FA levels were in the micromolar range. Note that 25 μM palmitate corresponds to nanomolar concentrations of unbound FA in BSA (0.1%)-containing medium (53).

Long Term Exposure of β-Cells to Palmitate Increases Susceptibility to Ca^{2+}-induced MPT Induction and Decreases Glucose-stimulated Insulin Secretion—Mitochondria from MIN6 cells treated with palmitate for 48 h (Fig. 2*A*), unlike controls, were completely depolarized by two 50 μM Ca^{2+} pulses, an effect that was largely reversed by the MPT inhibitor CsA. The effect of long term (48 h) palmitate exposure on INS-1 cells (Fig. 2*B*), which are more sensitive to MPT induction, was even more pronounced, with CsA unable to reverse the depolarization induced by Ca^{2+}. However, CsA provided protection when added before Ca^{2+}, demonstrating the classical nature of mitochondrial permeabilization in palmitate-treated INS-1 cells (Fig. 2*B*).

Mitochondrial depolarization can result from factors other than permeability transition. Unfortunately, methods based on fluorescent probe distribution tend to underestimate mitochondrial permeabilization (4). Thus, the effect of palmitate on MPT induction was further examined using mitochondrial Ca^{2+} uptake and respiration assays. Both Ca^{2+} uptake and oxygen consumption kinetics gave similar results to those found previously using fluorescent probes that monitor mitochondrial membrane potential (MIN6 cells, Fig. 3; INS-1 cells, data not shown), further demonstrating that mitochondria from β-cells exposed to palmitate were sensitized to MPT induction. Briefly, mitochondria from MIN6 and INS-1 β-cells exposed to palmitate for 48 h remained coupled; however, the ability of the mitochondrion to sequester Ca^{2+} was compromised (Fig. 3). Although FAs are known to be MPT co-inducers (48), it is unlikely that residual palmitate contributed to the observed effect, because an increase in BSA in the incubation medium from 0.1 to 0.5% did not diminish the difference between control and palmitate-treated cells (data not shown).

Long term exposure to palmitate has been shown to deteriorate GSIS in clonal and primary β-cells (54, 55), an effect that most likely proceeds through multiple pathways. In this work, exposure to palmitate led to an increase in basal insulin secre-

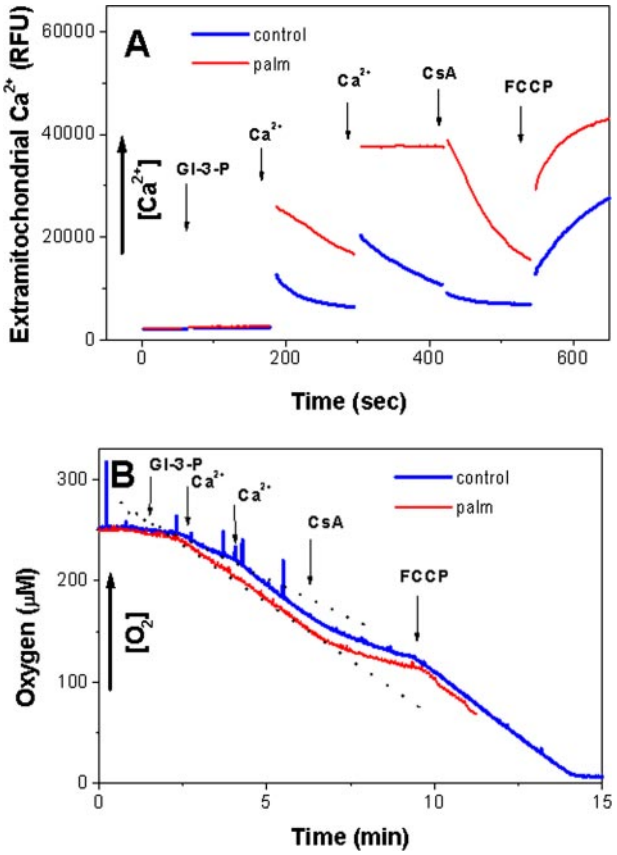

FIGURE 3. **Long term exposure to palmitate reduces mitochondrial Ca^{2+} accumulation capacity and abolishes respiratory control in the presence of Ca^{2+} in MIN6 cells.** *A,* MIN6 cells were treated with 0.5 mM palmitate (*palm*) for 48 h as described in Fig. 2. Extramitochondrial Ca^{2+} was monitored by fluorescence of calcium Green 5N (1 μM). Additions indicated by *arrows* are as follows: 7.5 mM glycerol 3-phosphate (*Gl-3-P*), 50 μM $CaCl_2$ (Ca^{2+}), 1 μM CsA, and 5 μM FCCP (respiratory uncoupler). Data shown are a typical representation of three independent experiments. *B,* respiratory measurements of control and palmitate-treated (0.5 mM for 48 h) MIN6 cells. Traces shown are representative of four independent experiments. *Dotted tangent lines* are used to emphasize the slope of the control and palmitate-treated traces, which show that abolition of respiratory control after the first Ca^{2+} pulse is partial in control cells but complete in those exposed to palmitate.

tion and inhibition of GSIS (Table 1, part B). These results were similar to what we have observed previously (47). Previous reports have shown that cyclosporin A inhibits GSIS in primary and clonal β-cells (56, 57), whereas the MPT inducer peripheral benzodiazepine receptor agonist PK11195 stimulates it (58), suggesting that glucose sensing is not unresponsive to the state of mitochondrial permeability. Similar to previous studies (56–58), CsA (5 μM) suppressed GSIS upon acute application. At the same time, FK-506, commonly used as a control for the MPT-independent effects of CsA (59, 60), did not exert a significant effect on secretion (Table 1, part A). In palmitate-exposed cells, GSIS was blunted compared with the control, and further reduced in response to CsA application (Table 1, part B).

Increased Susceptibility of β-Cells to MPT Induction after Long Term Palmitate Exposure Does Not Involve Ceramide- or ROS-related Pathways—The mechanism for the deleterious long term effects of palmitate on mitochondrial function is tissue- and cell-specific (48). In many cell types, elevated cellular formation of ceramides and ROS during exposure to palmitate are responsible for inhibition of mitochondrial function (61–63). To determine the potential contribution of ceramide and ROS formation in palmitate-induced MPT formation, we employed fumonisin B_1 and myriocin, inhibitors of the ceramide pathway (61), and the antioxidant *N*-acetylcysteine (NAC) (64). We also tested cyclic GMP application, which in some cases is able to protect against MPT opening (65), as well as GLP-1, which is reported to improve mitochondrial function in MIN6 cells (66) in a cAMP-dependent manner. In all cases, none of these agents mitigated MPT susceptibility in palmitate-exposed cells (Table 2). In the experiments involving NAC application, NAC was applied at the time of palmitate application, which was designed to alleviate oxidative stress during the long term 48 h of palmitate exposure. However, no change in MPT induction was observed (Table 2). However, it is also conceivable that long term exposure to FA causes stimulation of ROS production in permeabilized cells during experimental measurements (67, 68), which in turn can induce MPT (69). As such, we also tested the effect of NAC added directly to the assay medium. In this case, NAC did not provide a protective effect, nor did the other antioxidants Trolox (50 μM), MnTBAP (20 μM), or butyl-4-hydroxytoluene (50 μM) (data not shown).

Comparison of Saturated and Unsaturated FAs as Inducers of MPT—Depending on the cell type and conditions employed, saturated or unsaturated FAs can play a dominant role in lipotoxicity (48, 70, 71). In the literature, there are conflicting reports on the efficiency of palmitate (a saturated FA) and oleate (an unsaturated FA) to induce toxicity in β-cells. Similar efficiencies of these FAs (17) have been demonstrated, as well as a more dominating effect of palmitate over oleate (24, 25). The ability of a saturated FA (palmitate) and an unsaturated FA (oleate) to induce MPT upon acute and long term application to MIN6 cells is shown in Fig. 4, *A* and *B*, respectively. Opening of the MPT pore was estimated by Ca^{2+}-induced CsA-sensitive mitochondrial depolarization. Although both acute palmitate and oleate treatment promoted MPT opening (Fig. 4*A*), only palmitate significantly stimulated MPT after long term application (Fig. 4*B*). Considering that the free FA concentration of oleate in equilibrium with BSA is more than double that of

TABLE 1

The influence of CsA and FK-506 and palmitate on insulin secretion in clonal β-cells

In A, basal (at 1 mM glucose) and stimulated (at 20 mM glucose) insulin secretion were measured following a 1-h incubation of MIN6 cells in standard medium (see "Experimental Procedures"), containing either 5 μM CsA or 0.15 μM FK-506 (based on the known potency of these agents in inhibiting calcineurin) (59, 60). In B, basal and glucose-stimulated insulin secretion were measured after incubation of control and palmitate-treated cells with or without 5 μM CsA. Secretion was expressed as pg of insulin/μg of DNA, and the data represent the mean ± S.E. ($n = 3$).

A. Insulin secretion from untreated cells in the presence of CsA and FK-506

	Control	CsA	FK-506
Basal secretion	472 ± 32	596 ± 36	408 ± 50
Stimulated secretion	1936 ± 192	1346 ± 70[a]	1788 ± 100

B. Insulin secretion from control and palmitate-treated cells in the presence of CsA

	Control		Palmitate	
	−CsA	+CsA	−CsA	+CsA
Basal secretion	395 ± 41	340 ± 32	707 ± 58	466 ± 51[a]
Stimulated secretion	1889 ± 197	1171 ± 161[a]	1296 ± 104	901 ± 87[a]

[a] $p < 0.05$ *versus* control (secretion in the absence of CsA).

TABLE 2

Palmitate-induced sensitization to MPT was not prevented by the long term application of inhibitors of the ceramide pathway or other potential protectors of mitochondrial function

Mitochondrial susceptibility to permeability transition was estimated by CsA-sensitive mitochondrial depolarization in clonal MIN6 β-cells caused by 50 μM Ca^{2+} load (derived from $\Delta\Psi_m$ kinetic traces, $n = 3$). The values shown represent $\Delta\Psi_m$ as fluorescent safranin signal (% of that in control cells). Statistical analysis ($n = 3–4$) was performed using Student's *t* test. $\Delta\Psi_m$ in palmitate-treated cells was significantly ($p < 0.05$ marked with *) different from control, and all other experimental treatments were not significantly different from the cells treated with palmitate only.

	Control cells	Palmitate-treated cells	Palmitate + myriocin-treated cells	Palmitate + fumonisin B_1-treated cells	Palmitate + cGMP-treated cells	Palmitate + GLP-1-treated cells	Palmitate + NAC-treated cells
ΔΨ	100 ± 8	60 ± 13*	42 ± 5	51 ± 5	39 ± 14	44 ± 7	38 ± 12

palmitate (53), this differential ability of palmitic and oleic acids to induce MPT is even more remarkable. Examination of other FAs revealed a similarity between palmitate and stearate, and oleate and palmitoleate (see supplemental Fig. 1). It has been shown that unsaturated FAs differ from saturated FAs by their inability to stimulate the ceramide pathway (25, 72); however, as mentioned above, ceramide pathway inhibitors did not prevent the ability of palmitate to induce MPT pore opening (Table 2). Another important difference between saturated and unsaturated FAs is their opposing effects on membrane fluidity (72), which may alter the properties of MPT induction. However, the membrane fluidizing agent 2-(2-methoxyethoxy)ethyl 8-(*cis*-2-*n*-octylcyclopropyl) octanoate (A_2C) increased rather than decreased the effect of palmitate on MPT (see supplemental Fig. 2).

Surprisingly, we found that the combined long term application of palmitate and oleate to MIN6 cells, despite a higher total FA level, significantly attenuated mitochondrial sensitization to MPT pore induction compared with palmitate alone, with equimolar oleate largely counteracting palmitate induction of MPT (Fig. 4*C*). This phenomenon of oleate protection against palmitate cytotoxicity has been recently observed in clonal β-cells as well as in other cell lines in a PI 3-kinase-dependent manner (73, 74). Thus, we speculated that the apparent protective effect of oleate on MPT induction by long term palmitate exposure may also be PI 3-kinase-dependent. Indeed, we found that the protective effect of oleate was largely eliminated by the PI 3-kinase inhibitor LY294002 (but not its PI3-kinase-inactive structural analog LY303511), as well as another PI 3-kinase inhibitor wortmannin (Fig. 4*C* and Fig. 5*A*) (75). Application of LY294002 and wortmannin alone did not induce MPT in control cells as well as in cells treated only with palmitate (Fig. 5*B*). To further demonstrate PI 3-kinase involvement in the modulation of MPT by FA, we tested the effect of palmitate on phosphorylation of Akt (PKB), the main PI 3-kinase substrate (Fig. 5*C*). Insulin-stimulated phospho-Akt was used as the positive control (note that the moderate induction of Akt phosphorylation by insulin (1.4-fold, not shown) was likely because of the absence of a nutrient starvation period, as required by the protocol for palmitate exposure) (76). Fig. 5*C* shows that long term palmitate exposure in MIN6 cells suppressed phospho-Akt levels, which was counteracted by co-application of oleic acid. This supports the hypothesis that the PI 3-kinase/Akt pathway participates in MPT modulation by FA in β-cells and agrees with Akt involvement in palmitate lipotoxicity in β-cells (77) and myotubes (78).

Discrimination between Low and High Conductance MPT—Functional tests for MPT applied above were based on the passage of ions through the inner mitochondrial membrane, and therefore they do not discriminate between low and high conductance forms of MPT. As such, we sought to further characterize MPT opening in β-cells and determine whether MPT opening was of the low conductance or the high conductance forms. The classical swelling method for specific detection of high conductance MPT based on sucrose permeation into the matrix (4) cannot be applied to mitochondria in permeabilized β-cells. Consequently, we employed the citrate synthase assay for high conductance MPT, which was introduced by Korge and Weiss (33), and is based on acetyl-CoA permeation into the matrix followed by DTNB reduction by CoASH released in the course of the citrate synthase reaction as summarized in Fig. 6*A*. We first tested the applicability of this assay to mitochondria *in situ* with permeabilized hepatocytes (Fig. 6*B*), in which mitochondrial swelling can be monitored by cell suspension absorbance (79, 80) and correlated with the DTNB reduction rate as well as mitochondrial functional parameters. Hepatocytes were permeabilized with saponin according to the protocol applied to β-cells and loaded with Ca^{2+} in the presence of P_i. As expected, this caused immediate respiratory acceleration and a drop in mitochondrial membrane potential ($\Delta\Psi_m$) followed by mitochondrial swelling, which registered as a decrease in absorbance (Fig. 6*B*). After maximal swelling was achieved, the citrate synthase substrates were applied resulting in a sharp increase in absorbance, which indicated the reduction of DTNB (Fig. 6*B*), due to opening of the high conductance or full-size MPT pore. In the absence of Ca^{2+}, or in the presence of CsA, the inner membrane remained intact, and the citrate synthase reaction did not occur (data not shown), demonstrating that the citrate synthase assay allows for the specific detection of high conductance MPT in mitochondria *in situ*.

To characterize the mode of Ca^{2+}-induced MPT induction in β-cells, permeabilized MIN6 and INS-1 β-cells were subjected to a Ca^{2+} load sufficient to cause complete release of mitochondrial respiratory control and depolarization (Fig. 7, *top* and *middle traces*), and the permeability of the mitochondrial membrane was tested using the citrate synthase assay (*bottom trace*). This assay showed that the pore-forming agent alamethicin was required to make the matrix-associated citrate synthase available to extramitochondrial substrates. Thus, in these fully uncoupled β-cell mitochondria (which is characteristic of full-size MPT pores) the inner membrane remained impermeable to molecules the size of acetyl-CoA, indicative of a limited size pore. Unlike liver mitochondria, where such a situation occurs as a brief transient state before full-size MPT opening (7, 81–83), in β-cell mitochondria this state was sustained for at least 20 min.

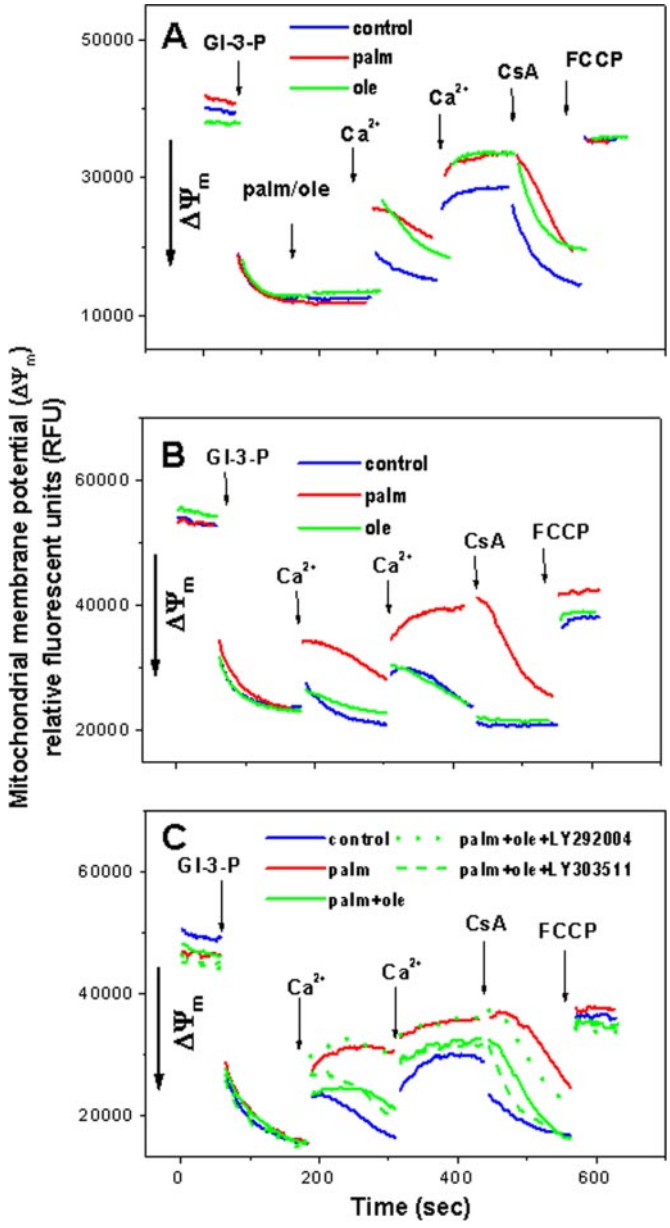

FIGURE 4. **Acute and long term effects of palmitate, oleate, and the PI 3-kinase inhibitor LY294002 on MPT induction by Ca^{2+} in clonal β-cells.** MPT induction was monitored by measuring mitochondrial membrane depolarization sensitive to CsA application after acute (*A*) and long term (48 h) exposure to FA (*B* and *C*). To test the acute effects of FA application, 25 μM palmitate (*palm*) or oleate (*ole*) was added to permeabilized MIN6 cells and assayed immediately. For long term FA application, intact MIN6 cells were treated with either 0.5 mM palmitate, or 0.1 mM oleate for 48 h, subsequently permeabilized, and then assayed. Mitochondrial membrane depolarization was quantified by measuring changes in safranin-O fluorescence. *Blue traces,* control cells; *red traces,* palmitate-treated cells; *green traces,* oleate-treated cells. Additions indicated by *arrows* are as follows: 7.5 mM glycerol 3-phosphate (*Gl-3-P*), 50 μM $CaCl_2$ (*Ca^{2+}*), 1 μM CsA, and 5 μM uncoupler FCCP. Both FAs stimulated MPT upon acute application (*A*). Only long term palmitate exposure resulted in MPT activation (*B*). Oleate partly prevented long term palmitate-induced MPT pore opening in a manner sensitive to the PI 3-kinase inhibitor LY294002 but not its structural inactive analog LY303511 (*C*). LY294002 or LY303511 was added to MIN6 cells at the time of FA addition at a final concentration of 50 μM. Data shown are representative of three individual experiments.

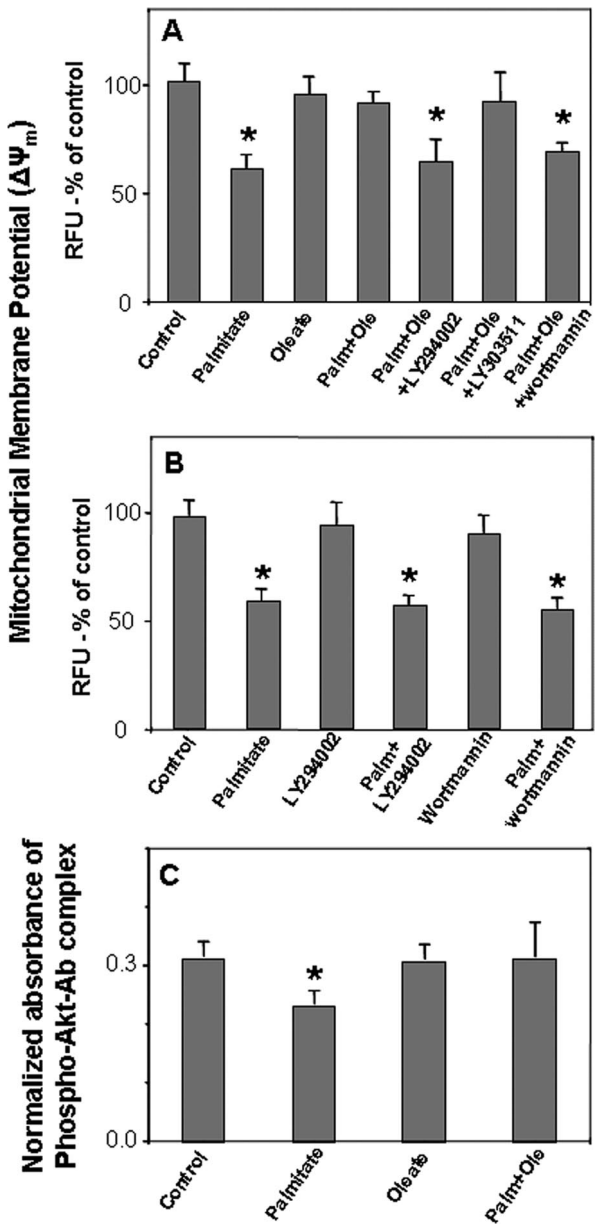

FIGURE 5. **Long term effect of palmitate and oleate on MPT induction in clonal β-cells and its relation to PI 3-kinase activation.** To estimate opening of MPT pore by Ca^{2+}-induced CsA-sensitive mitochondrial membrane depolarization, membrane potential of mitochondria loaded with 50 μM Ca^{2+} was quantified from kinetic traces of the type shown in Fig. 4, *B* and *C*. Thus, lower mitochondrial membrane potential under these conditions reflects increased MPT opening. *A,* 48-h exposure of MIN6 cells to palmitate (*palm*), but not to oleate (*ole*), resulted in increased Ca^{2+}-induced mitochondrial depolarization. When palmitate and oleate were added in combination, the presence of oleate protected MIN6 cells from calcium-induced mitochondrial membrane depolarization. In the presence of 50 μM LY294002, a PI 3-kinase inhibitor, the protective effect of oleate was removed, and mitochondrial membrane depolarization was achieved similar to that observed in palmitate-only treated cells. LY303511, an inactive analog of LY294002, was unable to remove the protective effect of oleate on mitochondrial membrane depolarization stimulated by palmitate. 100 nM wortmannin (*wort*), also a PI 3-kinase inhibitor, exerted an effect similar to that of LY294002. *B,* application of LY294002 or wortmannin to untreated control cells or to palmitate only treated cells had no impact on mitochondrial membrane potential. *C,* cells incubated with the same treatments as in *A* and *B* were analyzed for Akt phosphorylation as described under "Experimental Procedures." Phospho-Akt levels were expressed as absorbance of the phospho-Akt-antibody (*Ab*) complex normalized to absorbance of the total Akt-antibody complex and cell mass. Values represent the mean ± S.E. ($n = 3$); *, $p < 0.05$ *versus* control.

Because of the inability of Ca^{2+} to induce full-size MPT in β-cell mitochondria, we wanted to examine full-size MPT induction in β-cells induced by other mechanisms. In β-cell

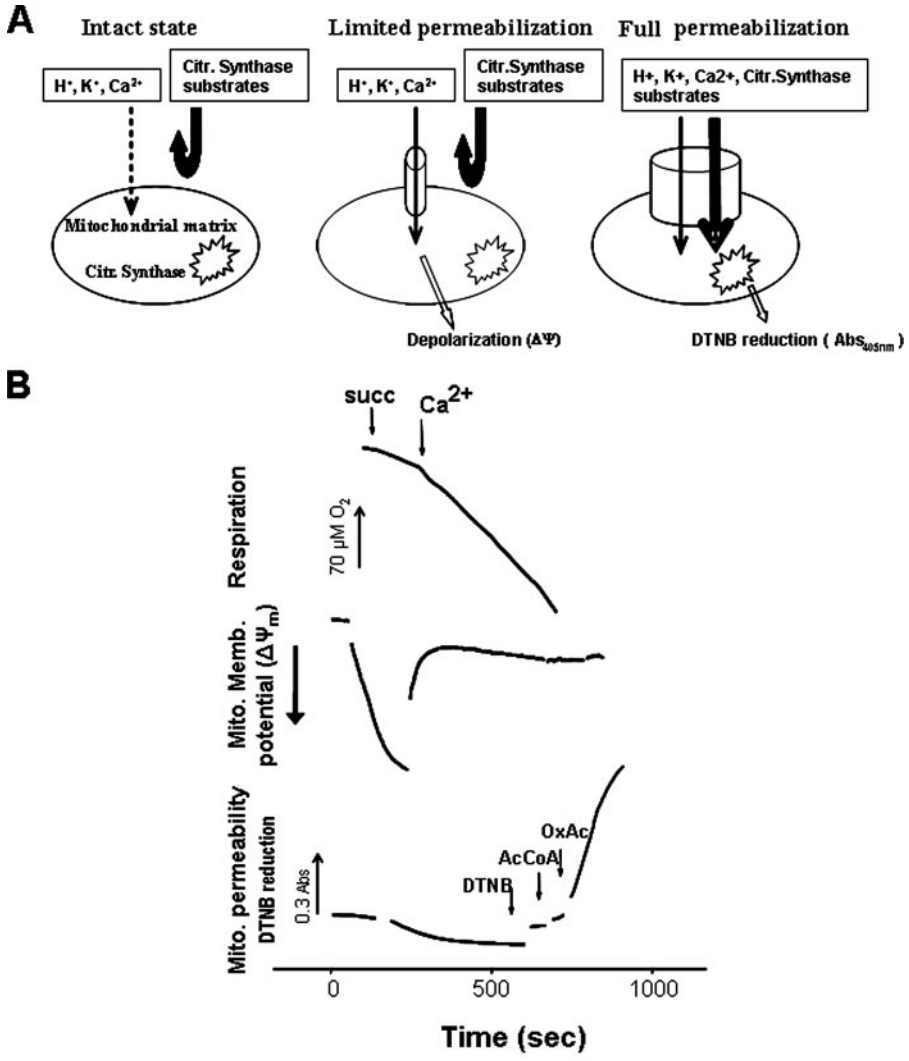

FIGURE 6. **Scheme illustrating methods for the detection of the low and high conductance MPT pore.** *A,* diagrammatic representation of the scheme for measuring low and high conductance MPT pores and its application to permeabilized hepatocytes. *B,* respiration (*upper trace*), mitochondrial membrane potential (*middle trace*), and absorbance (*lower trace*) of suspension of hepatocytes were monitored in parallel. Changes in absorbance reflect both mitochondrial swelling (the decrease in absorbance after Ca^{2+} addition) and DTNB reduction because of the citrate (*Citr.*) synthase reaction (the rise in absorbance after addition of citrate synthase substrates). Kinetic traces show that Ca^{2+}-induced respiratory acceleration and depolarization were followed rapidly by mitochondrial swelling, and full swelling corresponds to full accessibility of citrate synthase to acetyl-CoA. Permeabilized hepatocytes were suspended in the medium used for incubation of β-cells (0.60 mg of protein/ml, see "Experimental Procedures") containing 2.5 mM P_i. Additions indicated by *arrows*: 10 mM succinate (*succ*), 100 μM $CaCl_2$ (*Ca^{2+}*), 0.1 mM DTNB, 0.3 mM acetyl-CoA (*AcCoA*), and 0.5 mM oxaloacetate (*OxAc*). Data shown are representative of a typical experiment ($n = 3$).

mitochondria, commonly used hydroperoxides are poor inducers of the MPT pore, apparently because of slow utilization in the glutathione peroxidase reaction (57, 84). Consequently, to initiate high conductance MPT induction, we supplemented the Ca^{2+} load with the late product of oxidative stress, 4-HNE, which has been shown previously to act as an MPT co-inducer (85, 86). This supplementation caused formation of large pores in MIN6 mitochondrial membranes as indicated by an elevated rate of DTNB reduction in the presence of citrate synthase substrates (Fig. 8*A*).

To determine whether FA exposure affects full-size MPT opening in β-cells, MIN6 cells were exposed to long term application of a moderate concentration of palmitate (0.5 mM), which we have demonstrated stimulates Ca^{2+}-induced MPT (Fig. 2). This moderate FA application did not affect the ability of Ca^{2+} plus HNE to open the full-size MPT pore, although doubling the palmitate concentration to 1.0 mM resulted in increased opening of the high conductance MPT pore in the presence of Ca^{2+} plus HNE (Fig. 8*A*). Note that palmitate treatment alone (without subsequent HNE application; Fig. 8*A, dotted trace*) did not accelerate the citrate synthase reaction. Qualitatively, similar effects were observed between MIN6 and INS-1 cells (Fig. 8*B*). Finally, the effect of different palmitate concentrations on cell growth was estimated by the levels of cellular protein in cell suspension. Protein levels in cell preparations grown at moderate (0.5 mM) and high (1.0 mM) palmitate concentrations were 99 ± 9 and 68 ± 6% of control cell preparations, respectively ($n = 3, p < 0.05$). Thus, β-cells sensitized to low conductance MPT by application of a moderate concentration of palmitate maintained normal viability at the tested exposure times, whereas sensitization to high conductance MPT correlated well with increased cytotoxicity.

MPT in Intact β-Cells Exposed to Palmitate—In hepatocytes, myotubes, and thymocytes there is substantial similarity in the properties of MPT pores in permeabilized and intact cells (36, 87, 88). To assess the occurrence of MPT and its relation to FA treatment in intact β-cells, we initially applied the calcein/Co^{2+} technique for MPT detection (2). This, however, was made difficult because of poor quenching of cytosolic calcein fluorescence by Co^{2+}, likely because of the characteristically high level of Zn^{2+} in β-cells (89), which binds calcein tighter than Co^{2+} without fluorescent quenching (90). To circumvent this, MPT was examined in naive and palmitate-treated intact β-cells by measuring the effect of CsA on mitochondrial Ca^{2+} release and membrane potential. The first parameter, the CsA-dependent fraction of mitochondrial Ca^{2+} release, is used to characterize the rate of MPT pore-mediated mitochondrial Ca^{2+} efflux, whereas the second, CsA-induced mitochondrial hyperpolarization, is considered an indicator of steady-state MPT activity in intact β-cells (14, 36–38). For Ca^{2+} measurements, cells loaded with Fluo-4 accumulated 200–300 pmol of Fluo-4 per

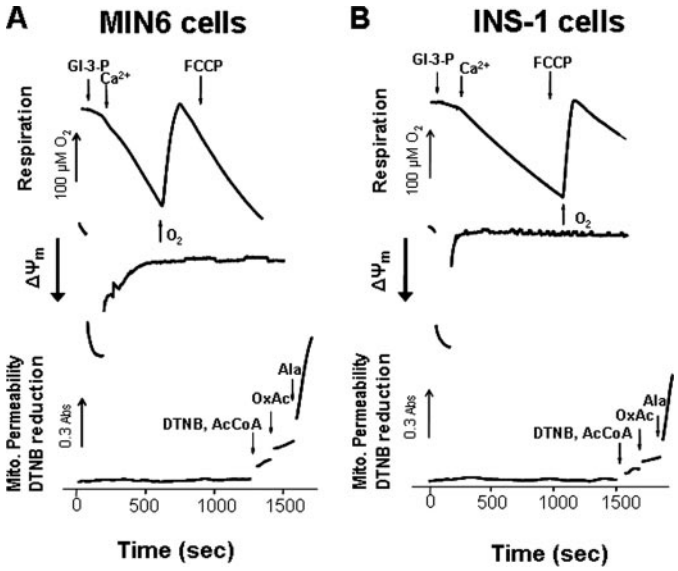

FIGURE 7. **Ca^{2+}-induced MPT in β-cells is characterized by high conductance and solute selectivity.** Kinetic traces show parallel monitoring of respiration (*upper traces*), mitochondrial (*Mito*) membrane potential (*middle traces*), and absorbance, reflecting inner membrane permeability (DTNB reduction because of the citrate synthase reaction, *lower traces*). Additions indicated by *arrows*: 7.5 mM glycerol 3-phosphate (*Gl-3-P*), 150 μM $CaCl_2$ (*Ca^{2+}*), 5 μM FCCP, 0.1 mM DTNB, 0.3 mM acetyl-CoA (*AcCoA*), and 0.5 mM oxaloacetate (*OxAc*). Mitochondria in MIN6 (*A*) and INS-1 (*B*) cells loaded with 150 μM Ca^{2+} underwent complete release of respiratory control (no further acceleration of respiration by uncoupler FCCP) and depolarization (*middle trace*) characteristic of high conductance permeability transition. However, monitoring of DTNB reduction (*lower trace*) demonstrated an absence of the citrate synthase reaction, gauged by an inability of oxaloacetate to induce a rise in absorbance. This indicates that the inner membrane remained impermeable to larger molecules for tens of minutes after pore opening. Final permeabilization of the inner membrane for larger molecules with alamethicin (*Ala*, 3 μM) demonstrates high citrate synthase activity in the matrix (*arrow* marked O_2 shows oxygen pulse provided to prevent anaerobiosis).

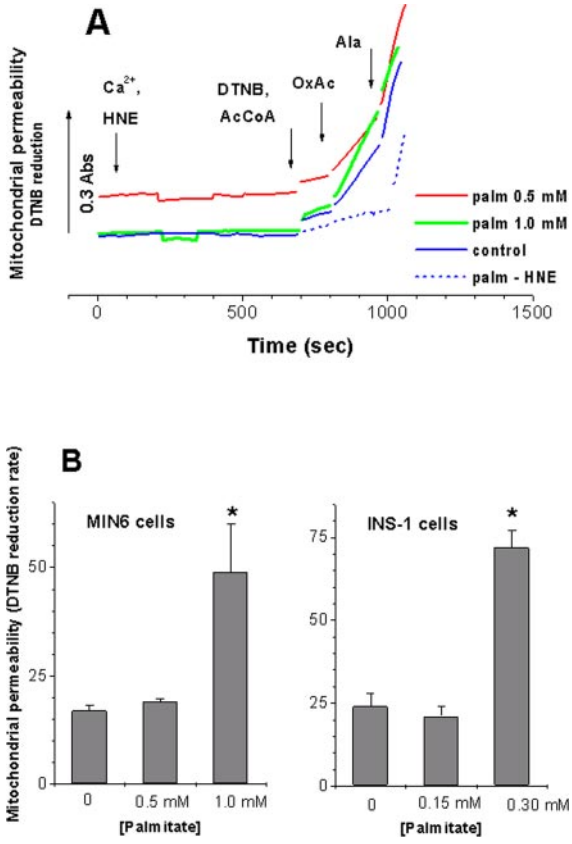

FIGURE 8. **High conductance MPT induced in β-cells by Ca^{2+}-HNE estimated by the citrate synthase assay.** *A,* full-size MPT pore opening was initiated by preincubation with Ca^{2+} (100 μM) in the presence of HNE (250 μM) and P_i (2.5 mM), followed by addition of citrate synthase substrates, and monitored by the rate of the citrate synthase reaction (DTNB reduction). Kinetic traces of the citrate synthase reaction in MIN6 cells (*blue,* control cells; *red,* cells exposed to 0.5 mM palmitate (*palm*) for 48 h; *green,* cells exposed to 1 mM palmitate for 48 h) show that exposure to a low concentration of palmitate (0.5 mM) sensitized mitochondria to limited size pore opening and had no sensitizing effect on full-size MPT pore opening, the activation of which requires a high palmitate level (1.0 mM). *Dotted blue trace* (cells exposed to 1.0 mM palmitate and preincubated with Ca^{2+} without HNE) shows that palmitate-exposed cells, like intact ones, do not open the full-size pore in response to Ca^{2+} load in the absence of oxidant. *B,* statistical analysis of mitochondrial membrane permeability as estimated by DTNB reduction in intact MIN6 and INS-1 cells exposed to low and high concentrations of palmitate for 48 h. Values represent the mean ± S.E. of three independent experiments.

mg of protein, which did not vary significantly between suspensions of control and palmitate-treated cells prepared in parallel. In Ca^{2+}-containing medium, leakage of Fluo-4 from β-cells catalyzed by drug-extruding transporters, such as the MDR pump, would increase the total fluorescent signal from cell suspensions thereby mimicking a rise in cytosolic Ca^{2+}. To evaluate this effect we used cyclosporin H, which inhibits Fluo-3 efflux driven by the MDR pump (91). Control kinetic traces (Fig. 9*A, panel ii*) show no effect of cyclosporin H on probe fluorescence, which indicates negligible probe efflux via the MDR pump in the time frame tested. The potential contribution of the β-cell organic anion exchange activity to the observed fluorescence was examined using 1 mM probenecid (42, 43), which also demonstrated a negligible contribution of these transporters on the loading of these fluorescent probes into the cells (data not shown).

Cellular sarco/endoplasmic reticulum Ca^{2+}-ATPase-dependent stores were first emptied with thapsigargin (1 μM), and then Ca^{2+} loaded into the mitochondrial matrix was liberated by depolarizing the mitochondria with FCCP. This was preceded by oligomycin treatment to prevent ATP synthase reversal (Fig. 9*A*). Preincubation with CsA (but not FK-506) for 5 min partially inhibited Ca^{2+} release, indicating that Ca^{2+} was released from mitochondria through the MPT pore as well as by other mechanisms (reversal of Ca^{2+} uniporter, Ca^{2+} exchangers). These results are similar to what is found in thymocytes (36) and brain cells (37). The contribution of MPT to mitochondrial Ca^{2+} release was stimulated by exposure to palmitate (Fig. 9, *A* and *C*), much like in permeabilized cells.

Loading of β-cell suspensions with rhodamine 123 created an intracellular pool of the dye, which in a short term experiment responded to changes in mitochondrial membrane potential and not plasma membrane potential, which was evidenced by a test with 50 mM KCl (92). Inhibition of the MDR pump by cyclosporin H (5 μM) during 10–15-min incubations did not alter cellular fluorescence of rhodamine 123 (supplemental Fig. 3), thus showing its applicability for testing $\Delta\Psi_m$ in this system. A minor effect of oligomycin on Ψ_m in control and palmitate-treated cells (supplemental Fig. 3) indicated that mitochondrial membrane potential was maintained by respiration rather than by hydrolysis of glycolytic ATP (92). Application of CsA (but not FK-506) to cell suspensions led to an

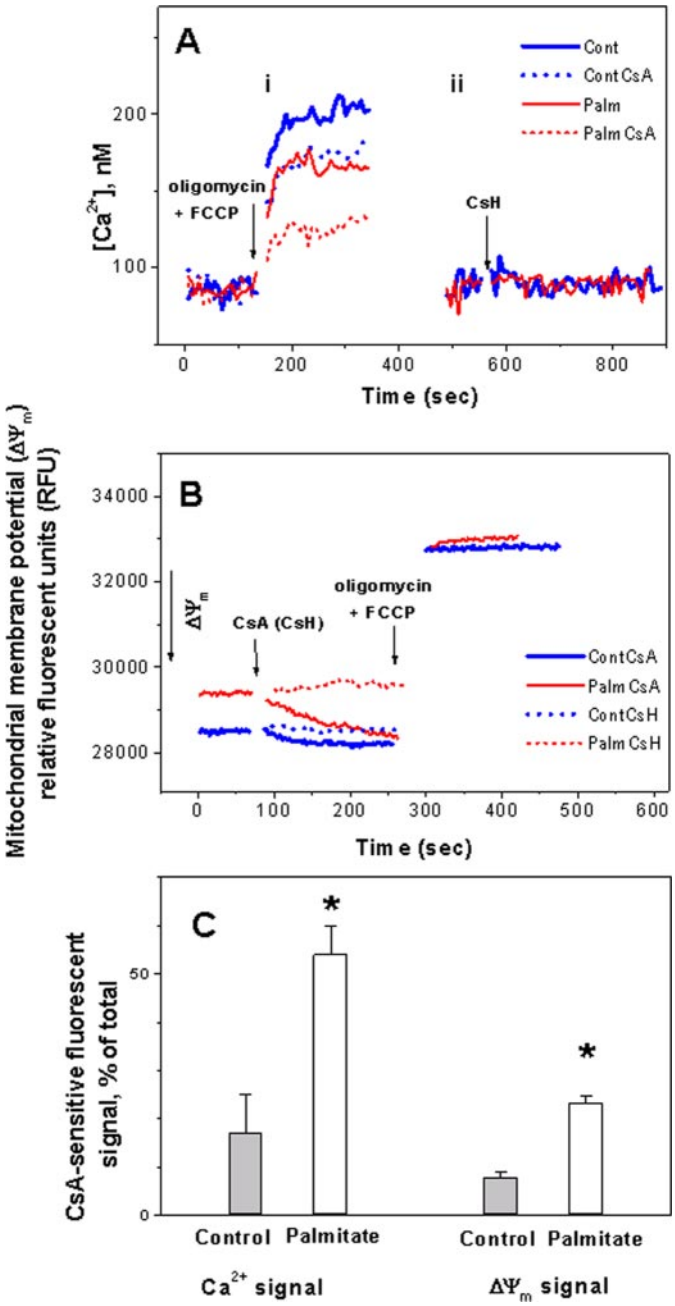

FIGURE 9. **Long term exposure to palmitate increases the CsA-sensitive fraction of the mitochondrial Ca^{2+} pool and stimulates CsA-induced mitochondrial hyperpolarization in intact MIN6 cells.** *A,* MIN6 control cells and cells exposed to 0.5 mM palmitate (*palm*) for 48 h were loaded with Fluo-4 and suspended in KRB/BSA buffer. Cells were treated with thapsigargin (1 μM) to empty endoplasmic Ca^{2+} stores, after which (as shown in the *left subpanel i*) Ca^{2+} sequestered in mitochondria was released by the application of FCCP (5 μM) + oligomycin (3 μM) (marked by *arrow*) in the absence or presence of CsA (5 μM, added 5 min prior to uncoupler FCCP). *Right subpanel ii* shows that CsH (5 μM), an MPT-inactive but MDR pump-inhibiting CsA analog, has negligible effects on fluorescence of Fluo-4-loaded cells. Data shown are representative of three independent experiments. *B,* representative traces of mitochondrial membrane potential in MIN6 cells ($n = 3$) loaded with rhodamine 123. After a short incubation, cells were treated with CsA (5 μM) and subsequently with FCCP (5 μM) + oligomycin (3 μM) (*solid kinetic traces*). Alternatively, to determine potential fluorescent alteration caused by inhibition of MDR pump by CsA, CsH (5 μM) was applied instead of CsA (*dotted traces*). *C,* statistical analysis of the effect of CsA on the release of mitochondrial Ca^{2+} and mitochondrial membrane potential in control and palmitate-treated cells. Values represent the mean ± S.E. ($n = 3$); *, $p < 0.05$ *versus* control.

increase in mitochondrial polarization (Fig. 9, *B* and *C*). As a control for the potential inhibitory effect of CsA on MDR pump-dependent rhodamine 123 extrusion, the MPT-inactive MDR-inhibiting analog CsH was applied (2, 37), which had no substantial effect on fluorescent kinetics (Fig. 9*B*, *dotted lines*). Similar CsA-induced elevation of $\Delta\Psi_m$, indicating cessation of transient activity of MPT, which causes fractional mitochondrial depolarization, was observed in several other cell types (14, 37). After application of CsA mitochondrial potential was dissipated by FCCP (+oligomycin). Data in Fig. 9, *B* and *C*, show that the CsA-dependent $\Delta\Psi_m$ increase in palmitate-exposed cells constitutes a greater portion of the total signal than in control cells. Overall, sensitization to MPT by exposure to palmitate observed in permeabilized cells agrees with manifestations of basal MPT activity found in intact cells.

DISCUSSION

A Solute Selective Form of MPT in β-Cells—Low conductance MPT was initially defined in terms of pore size allowing passage of only ions, such as H^+, K^+, Ca^{2+}, but not larger molecules, including sucrose and nucleotides (6, 7). This type of MPT can occur either as a brief, transient state between closed and the fully open MPT pore or as a separate long lasting condition. In other cell types, when the long lasting low conductance MPT pore was examined functionally, it produced a modest mitochondrial depolarization and only slight, if any, acceleration of respiration (13, 93–96). However, a distinctive property of Ca^{2+}-induced MPT in clonal β-cells is that despite its narrow pore diameter, conductivity is sufficient for the maximal dissipation of membrane potential and acceleration of respiration, *i.e.* complete release of respiratory control. The only other published example of this particular phenomenon concerns the narrow MPT pore seen in liver mitochondria after closure of the high conductance MPT pore (97). Interestingly, this variant of the MPT pore in liver was insensitive to CsA and ADP, as well as to Ca^{2+} and the removal of FAs. In contrast, the MPT pore in β-cells opened directly in response to Ca^{2+} and responded to CsA and ADP. Unlike the low conductance pores reported in liver mitochondria (93), the narrow pore in β-cells was initiated specifically by Ca^{2+} (Sr^{2+} was unable to substitute for Ca^{2+}). In contrast to low conductance pores in ascites mitochondria (6), pores in β-cells responded to Ca^{2+} load but not ΔpH on the inner membrane because P_i, which reduces the proton gradient across mitochondrial membranes, stimulated permeabilization. MPT in its fully open form is usually considered a trigger of cell death (2, 5, 98). The potential role of its intermediate forms in the regulation of cellular metabolism (1, 6, 14, 98) has not been extensively studied. The possibility of sustained opening of a highly conductive small sized pore, as shown here in clonal β-cells, provides evidence for additional roles of MPT in cell physiology.

Long Term Effect of Saturated FA on MPT and Possible Mechanisms—Previous work reporting the pro-apoptotic effect of saturated FA (72, 100, 101) was performed on intact cardiomyocytes and β-cells; however, mitochondrial depolarization (which is considered an indicator of MPT) was observed under poorly defined intracellular conditions. In particular, after exposure to FA, intact cells retained elevated

intracellular FA, which could partly account for the observed effects. Moreover, FAs exert both acute (direct) and long term (indirect) effects on MPT (70). In intact cells, both actions affect the mitochondria simultaneously. In this study, palmitate stimulated mitochondrial permeabilization both acutely and chronically, whereas oleate stimulated MPT acutely yet provided protection against MPT opening after long term exposure. This time-dependent effect could partly explain controversial results in this field, including reports that oleate both stimulates (17) and protects (24, 25) against MPT and cell death. Additionally, permeabilization of FA-treated cells allowed removal of intracellular FA, such that mitochondria in the control and FA-treated cells were studied in identical FA-free medium and the long term effects of FA could be observed in a pure state. Under these conditions, cell exposure to saturated FA was shown to produce intrinsic alterations in mitochondria predisposing them to Ca^{2+}-induced permeabilization.

A remarkable feature of the long term effect of FA exposure on MPT in β-cells is its dependence on FA saturation, despite the fact that saturated and unsaturated FAs exert similar effects acutely. One essential distinction between saturated and unsaturated FA is their opposite effects on membrane fluidity, which can be related to the occurrence of MPT. However, the membrane-fluidizing agent 2-(2-methoxyethoxy)ethyl 8-(*cis*-2-*n*-octylcyclopropyl) octanoate did not reverse the activation of MPT induced by palmitate (supplemental Fig. 2). Another characteristic property of saturated FAs is the stimulation of ceramide biosynthesis, which can affect mitochondrial properties (102). Nevertheless, inhibitors of ceramide formation, fumonisin B_1 and myriocin, were unable to protect against mitochondrial sensitization to permeability transition induced by palmitate. Several antioxidants also had no effect. It is unlikely that the recently reported direct permeabilizing action of the Ca^{2+}-palmitate complex on the mitochondrial membrane (103) plays a role in the long term effect of palmitate on MPT pore induction, especially because the latter effect is sensitive to CsA.

Oleate provided significant protection from palmitate-induced MPT through a mechanism sensitive to inhibition of the PI 3-kinase pathway. This was shown using structurally and mechanistically distinct PI 3-kinase inhibitors LY294002 and wortmannin, providing good indication for the involvement of this kinase (104). Further measurement of phosphorylation of Akt, the PI 3-kinase downstream target, supports this conclusion and indicates that the PI 3-kinase/Akt pathway is involved in modulation of mitochondrial permeability state by FAs in β-cells. It has recently been found that palmitate and oleate exert opposite effects on cell death through an interaction involving the PI 3-kinase (73, 74). In particular, protection against palmitate-induced apoptosis in clonal β-cells provided by unsaturated FA application was PI 3-kinase-dependent (73). In cardiomyocytes, oxidative stress-induced apoptosis was prevented by insulin in a PI 3-kinase/Akt-dependent manner (105), and the cytoprotective effect of PI 3-kinase signaling in this cell type involved enhancement of the mitochondrial ability to accumulate Ca^{2+} without undergoing terminal depolarization (106). Our data indicate that this pathway also controls

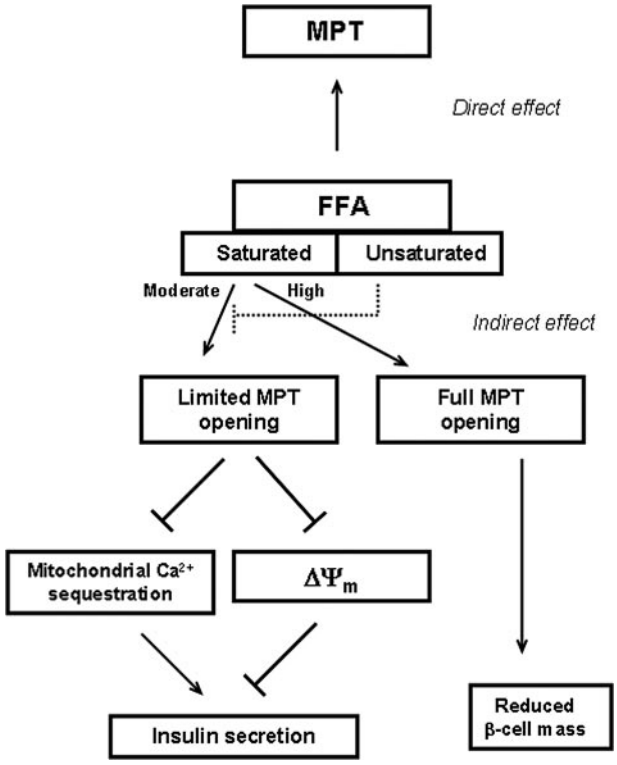

FIGURE 10. **Scheme illustrating potential short and long term effects of FA on MPT in β-cells.** Upon acute application, both saturated and unsaturated FAs stimulate MPT pore opening. After a long term (48 h) exposure to a low, noncytotoxic concentration of saturated FAs, mitochondria become sensitized to opening of the limited size MPT pore (with high conductance properties; specific to β-cells), whereas unsaturated FAs exert a protective effect that is PI 3-kinase-dependent. Stimulation of full-size MPT pore opening is brought about by exposure to a higher concentration of FA, which is associated with a cytotoxic effect and could contribute to reduced β-cell mass. Opening of the limited size MPT pore in β-cells displays unique properties compared with limited size pores in other tissues. β-Cell limited size MPTs allow only the passage of small molecules but promote reduction in mitochondrial membrane potential, uncouple mitochondrial respiration, and prevent mitochondrial calcium uptake. These events could have opposing effects on insulin secretion. Decreased mitochondrial calcium uptake could promote insulin secretion by maintaining high cytosolic calcium, which is required for insulin secretion. Conversely, decreased mitochondrial membrane potential, despite the faster rate of respiration, uncouples respiration from ATP production. As a consequence, decreased ATP production would inhibit insulin secretion. Clearly, more studies are required to further elucidate the role of limited size MPT pores on β-cell function.

the limited opening of the mitochondrial pore, in addition to the full-size form of MPT. Interestingly, oleate stabilized mitochondria in β-cells specifically against Ca^{2+} load, because similar exposure to oleate, conversely, sensitized mitochondria to subsequent loading with FA (68).

Activation of MPT and Potential Implications for β-Cell Physiology—A moderate level of saturated FA applied within a limited time frame activated limited size MPT opening without any effect on cell viability. Data from intact cells demonstrated that basal MPT activity is associated with lowered $\Delta\Psi_m$ and facilitated release of mitochondrial Ca^{2+}. It is conceivable that this could have opposing effects on β-cell function. First, mitochondrial de-energization (depolarization) caused by MPT activation could slow down ATP synthesis and impede insulin secretion and, in doing so, potentially compromise β-cell function. On the other hand, the limitation of mitochondrial Ca^{2+} uptake as a result of activated MPT could stimulate insulin

release through increased cytosolic Ca^{2+} levels that would counteract the effect of mitochondrial de-energization. Such a possibility is supported by a recent report in which the early phase of MPT-related β-cell apoptosis was accompanied by increased insulin secretion in association with elevated cytosolic Ca^{2+} (58). Finally, a moderate activation of MPT could exert such beneficial effects as the release of excessive matrix Ca^{2+}, acceleration of mitochondrial respiration, and moderation of ROS production (3). In particular, it was suggested that a certain level of MPT activity is required for optimal insulin secretion (56). Thus, it is likely that a fine balance between these factors and specific metabolic conditions would determine the final outcome of FA-induced activation of limited size MPT on β-cell function. A higher level of saturated FA would sensitize β-cells to the opening of the large size variant of MPT leading to cytotoxic effects.

A report questioning the contribution of oxidative stress in lipid-induced deterioration of β-cell function (99) suggested that lipotoxic damage of insulin-secreting cells may occur through different (or less oxidative stress-dependent) mechanisms. As demonstrated in this study, sequential activation of different forms of MPT by saturated FA could be one of the alternative mechanisms underlying β-cell lipotoxicity. The scheme depicted in Fig. 10 illustrates the potential contribution of FA-stimulated MPT in β-cell cytotoxicity. Obviously, further studies are needed to elucidate specific contributions of variable mitochondrial permeability on the regulation of insulin secretion. Current understanding of the role of MPT in β-cell pathophysiology is based on observations suggesting that MPTs induced by cytokines (15) and FAs (17) participate in promoting β-cell death. The present data demonstrate that the role of MPT is more versatile. Limited mitochondrial pore opening caused by a moderate FA load could modulate β-cell metabolism, essentially without changing viability, thereby contributing to β-cell dysfunction and the initial steps in the pathogenesis of T2D.

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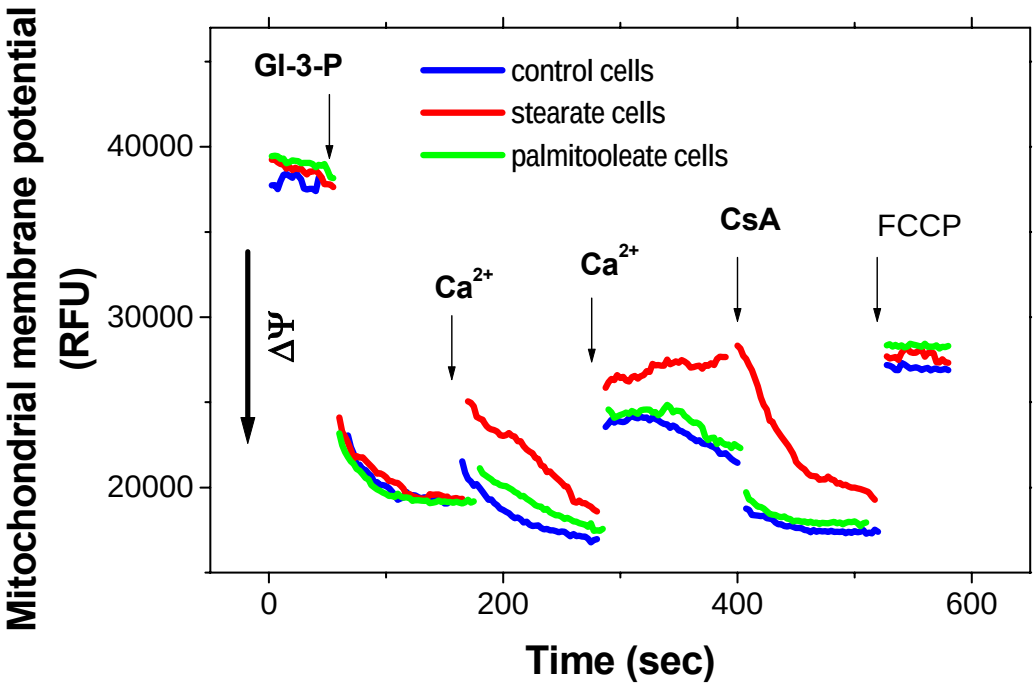
SUPPLEMENTAL FIGURES

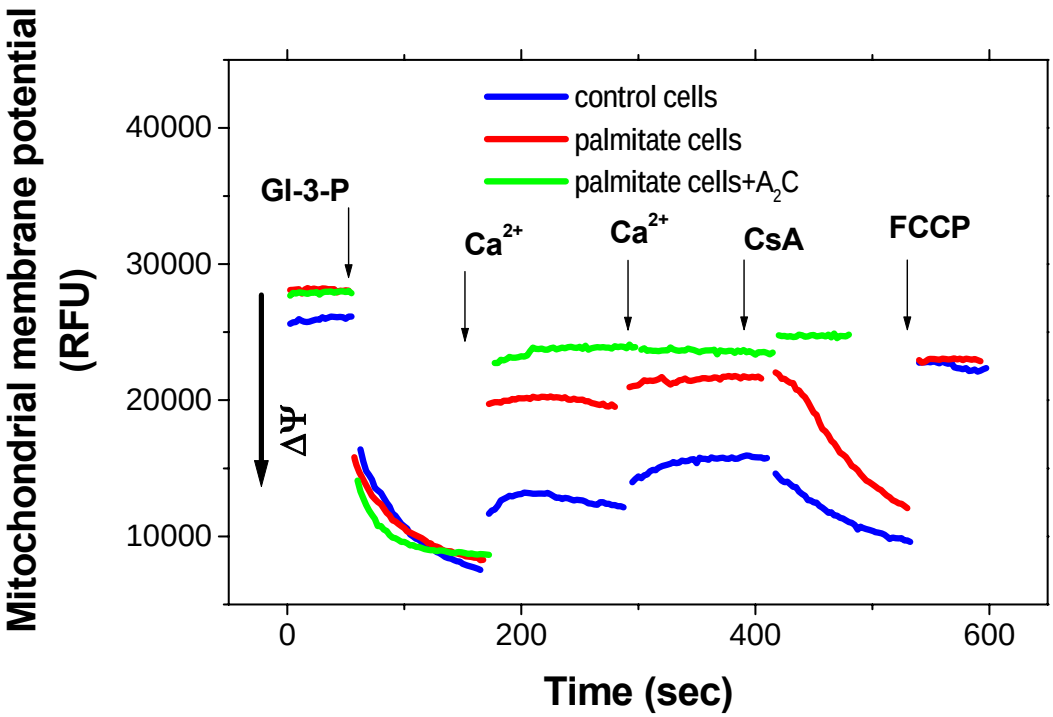
Fig. S1. Long-term effects of stearate and palmitoleate on MPT induction by Ca^{2+} in clonal β-cells. Stearate (0.5 mM) causes MPT activation upon 48 h exposure and removal of the FA, while the same concentration of palmitoleate exerts only a minor effect. MPT dynamics was monitored by mitochondrial membrane potential, blue traces – control cells, red traces–stearate-treated cells, green traces–palmitoleate-treated cells.

Fig. S2. The membrane-fluidizing agent A_2C does not prevent sensitization to MPT caused by exposure of MIN6 cells to palmitate. Assays were performed as described in the Fig.2, membrane fluidizing FA derivative A_2C was applied at a concentration of 125 nmol/mg protein.

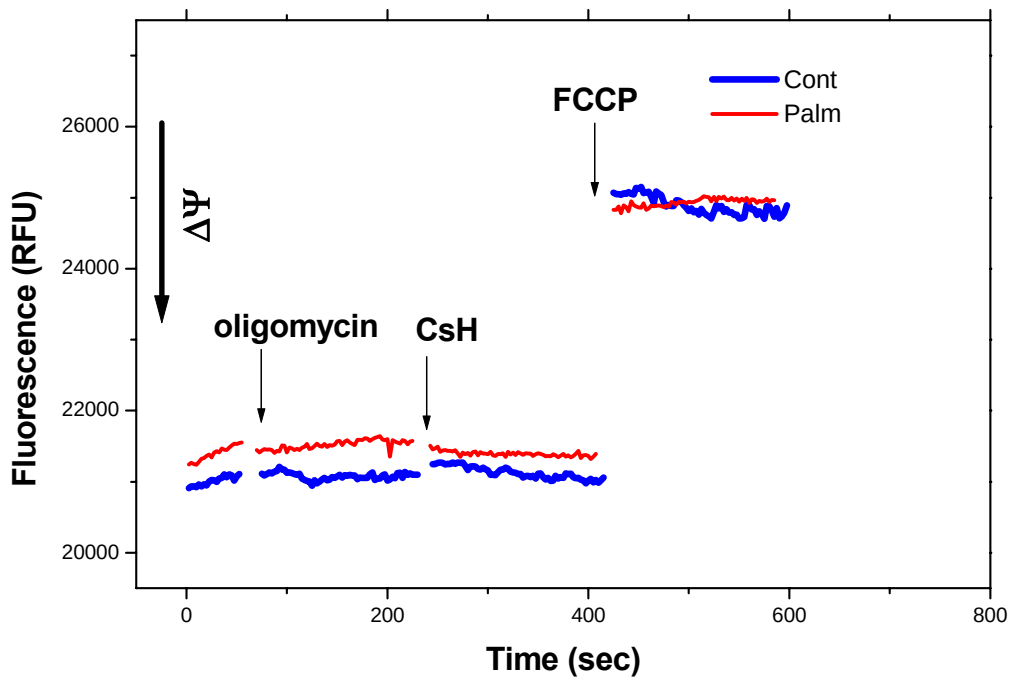
Fig. S3. Rhodamine 123 fluorescent signal from MIN6 cells is essentially independent on inhibitors of F_0F_1 ATPase (oligomycin) and MDR pump (cyclosporine H). Control (blue trace) and palmitate-exposed (red trace) cells loaded with rhodamine 123 were treated with oligomycin (3 μM) and cyclosporine H (5 μM), finally $\Delta\Psi$ was dissipated with FCCP (5 μM). Kinetics shown are representative of three independent experiments. (n = 3).


Mitochondrial membrane potential (RFU)
40000
30000
20000
0
200
400
600
Time (sec)
Gl-3-P
control cells
stearate cells
palmitooleate cells
ΔΨ
Ca2+
Ca2+
CsA
FCCP

Suppl. Fig.1.


Mitochondrial membrane potential (RFU)
40000
30000
20000
10000
0
200
400
600
Time (sec)
control cells
palmitate cells
palmitate cells+A2C
Gl-3-P
Ca2+
Ca2+
CsA
FCCP
ΔΨ

Suppl.Fig. 2.


Fluorescence (RFU)
26000
24000
22000
20000
0
200
400
600
800
Time (sec)
ΔΨ
oligomycin
CsH
FCCP
Cont
Palm

Suppl.Fig. 3.

Limited Mitochondrial Permeabilization Is an Early Manifestation of Palmitate-induced Lipotoxicity in Pancreatic β-Cells

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