Effects of Secretagogues and Bile Acids on Mitochondrial Membrane Potential of Pancreatic Acinar Cells

COMPARISON OF DIFFERENT MODES OF EVALUATING $\Delta\psi_m$*

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In this study, we investigated the effects of secretagogues and bile acids on the mitochondrial membrane potential of pancreatic acinar cells. We measured the mitochondrial membrane potential using the tetramethylrhodamine-based probes tetramethylrhodamine ethyl ester and tetramethylrhodamine methyl ester. At low levels of loading, these indicators appeared to have a low sensitivity to the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone, and no response was observed to even high doses of cholecystokinin. When loaded at high concentrations, tetramethylrhodamine methyl ester and tetramethylrhodamine ethyl ester undergo quenching and can be dequenched by mitochondrial depolarization. We found the dequench mode to be 2 orders of magnitude more sensitive than the low concentration mode. Using the dequench mode, we resolved mitochondrial depolarizations produced by supramaximal and by physiological concentrations of cholecystokinin. Other calcium-releasing agonists, acetylcholine, JMV-180, and bombesin, also produced mitochondrial depolarization. Secretin, which employs the cAMP pathway, had no effect on the mitochondrial potential; dibutyryl cAMP was also ineffective. The cholecystokinin-induced mitochondrial depolarizations were abolished by buffering cytosolic calcium. A non-agonist-dependent calcium elevation induced by thapsigargin depolarized the mitochondria. These experiments suggest that a cytosolic calcium concentration rise is sufficient for mitochondrial depolarization and that the depolarizing effect of cholecystokinin is mediated by a cytosolic calcium rise. Bile acids are considered possible triggers of acute pancreatitis. The bile acids taurolithocholic acid 3-sulfate, taurodeoxycholic acid, and taurochenodeoxycholic acid, at low submillimolar concentrations, induced mitochondrial depolarization, resolved by the dequench mode. Our experiments demonstrate that physiological concentrations of secretagogues and pathologically relevant concentrations of bile acids trigger mitochondrial depolarization in pancreatic acinar cells.

Pancreatic acinar cells are polarized epithelial cells responsible for synthesis and secretion of digestive enzymes. The apical part of these cells contains a high density of secretory granules, whereas the basal region contains a well developed endoplasmic reticulum and the nucleus (1–3). Thin ER[1] strands linked to the main basal ER can be found in the apical region (3, 4). Pancreatic acinar cells contain a high density of mitochondria. Approximately 8% of the cell volume is occupied by these organelles (1). Mitochondria are positioned in three distinct groups: perigranular (also termed "mitochondrial belt"), perinuclear, and subplasmalemmal (5–8).

Secretagogues (*e.g.* the circulating hormone CCK) utilize a calcium signaling cascade to trigger and regulate enzyme and fluid secretion in pancreatic acinar cells (reviewed in Refs. 9–12). The secretagogue-induced calcium rise occurs primarily due to calcium release from internal stores. The stores are replenished by calcium influx through the basal portion of the plasma membrane and the tunneling action of the endoplasmic reticulum (13–15). The majority of calcium signals, induced by physiological doses of agonists, are confined to the apical region of the cell (local calcium signals), but even at physiological doses of CCK, some calcium responses originate in the apical region but propagate to the basal part (*i.e.* become global) (16–19). Whereas transient local and global calcium signals are essential for the functioning of the cells, sustained global calcium elevations, which could be triggered by supramaximal doses of agonists or by bile acids, are highly detrimental (20–22). It has been hypothesized that such calcium toxicity is responsible for the damage to pancreatic tissue during acute pancreatitis (23). This hypothesis recently received considerable experimental support (20–22).

Mitochondria in pancreatic acinar cells accumulate calcium during cytosolic calcium responses (5, 6, 24) and play an important role in shaping calcium transients (5, 6, 24, 25). In particular, the perigranular mitochondrial belt helps to restrain calcium signals to the apical region of the cell (5, 6, 8, 24).

Important roles for calcium in stimulus-metabolism coupling (26) (see also reviews in Refs. 27–29) and in mitochondria-mediated cell pathology (reviewed in Refs. 29–31) have been documented for a number of cell types. We have recently characterized the correlation between cytosolic calcium, mitochondrial calcium, and mitochondrial NADH production in pancreatic acinar cells (32). Specifically, we found that even relatively short (a few seconds) apical calcium signals are able to produce a considerable increase of mitochondrial NADH. It is important

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[1] The abbreviations used are: ER, endoplasmic reticulum; CCK, cholecystokinin; ACh, acetylcholine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; JMV-180, synthetic CCK analogue JMV-180 (Boc-Tyr(SO₃H)-Nle-Gly-Trp-Nle-Asp-2-phenylethylester; TLC-S, taurolithocholic acid 3-sulfate; TCDC, taurochenodeoxycholic acid; TDC, taurodeoxycholic acid; TC, taurocholic acid; TMRE, tetramethylrhodamine ethyl ester; TMRM, tetramethylrhodamine methyl ester; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid).

to note that, depending on the status of the mitochondria, both positive and negative changes of NADH concentrations could be induced by calcium signals (32). Both positive and negative NADH changes were also recorded in whole cell patch clamp experiments when the patch pipette solution contained 2 mM of ATP, suggesting that these changes are not due to fluctuations of ATP concentration but, most probably, due to a direct effect of calcium on the mitochondria.

The mitochondrial membrane potential ($\Delta\psi_m$) is a very important parameter, controlling different aspects of mitochondrial metabolism and ultimately ATP production. Our previous attempts to resolve changes of mitochondrial membrane potential induced by calcium-releasing secretagogues were unsuccessful (22). These attempts were undertaken using loading of the cells with a low concentration of a mitochondrial probe (tetramethylrhodamine methyl ester (TMRM)). In the current study, we decided to employ both the conventional low concentration and a high concentration dequench mode for evaluation of the mitochondrial membrane potential. The two modes of measurements are based on different principles. The low concentration mode relies on the Nernst distribution of the positively charged, yet membrane-permeable, fluorescent indicator between cytosol and mitochondria. Depolarization of mitochondria should result in redistribution of the indicator from the mitochondria to the cytosol, causing a decrease of mitochondrial fluorescence and increase of cytosolic fluorescence. The dequench mode requires high concentrations of indicators (tetramethylrhodamine ethyl ester (TMRE) or TMRM in our experiments). When loaded at high concentrations, these probes further concentrate in the negatively charged mitochondria and, as a result of such dense packaging, become quenched. Depolarization of the mitochondria results in release of the indicator from mitochondria to the cytosol, dequench of the indicator, and therefore an overall increase of cell fluorescence (for a detailed review of the techniques of $\Delta\psi_m$ measurements and techniques for studies of other mitochondrial functions, see Ref. 33). It is important to note that complete dequench of an indicator does not necessarily mean complete dissipation of $\Delta\psi_m$. Mitochondria could still be substantially hyperpolarized when dequench response reaches its maximum (33).

The first aim of our study was to characterize the relative sensitivities of the low concentration and the dequench mode of evaluation of changes in $\Delta\psi_m$. Thereafter, we used the more sensitive of the two methods to identify and investigate $\Delta\psi_m$ changes, induced by physiological and supramaximal doses of CCK and other calcium-releasing agonists. We also compared the effects of CCK with that of the calcium-releasing agonist JMV-180, which cannot induce a sustained toxic calcium elevation (34, 35) and with the secretagogue secretin, employing cAMP rather than Ca^{2+} as a second messenger (36, 36–38). The final aim of this investigation was to elucidate the effect of putative activators of acute pancreatitis, namely bile acids, on $\Delta\psi_m$.

MATERIALS AND METHODS

Cell Preparation—Pancreata were obtained from adult mice (CD1) killed by cervical dislocation in accordance with the Animal (Scientific Procedure) Act of 1986. Pancreatic acinar cells were prepared by injecting 1 ml of 200 units ml^{-1} collagenase CLSPA (Worthington) and digesting for 16–17 min at 37 °C with permanent agitation. After digestion, the pancreas was agitated manually to release single cells or small clusters in solution. Cells were washed three times by centrifugation in standard "extracellular" solution. All experiments were performed at room temperature (23–25 °C), and cells were used within 3–4 h after isolation. During experiments, the cells were placed on a glass coverslip coated with poly-L-lysine (0.01%), which was attached to an open perfusion chamber.

Solutions—The standard extracellular solution, used for cell preparation and for perfusion of cells during experiments, contained 140 mM

NaCl, 4.7 mM KCl, 1.13 mM MgCl$_2$; 1 mM CaCl$_2$; 10 mM D-glucose; 10 mM Hepes (adjusted to pH 7.2 by NaOH). Solutions with the required concentrations of CCK, ACh, secretin, dibutyryl cyclic AMP, bombesin, and bile acids were prepared by dissolving these compounds in the extracellular solution. CCCP was initially prepared as a 10 mM stock solution in ethanol and then diluted in the extracellular solution before beginning experiments; the concentration of ethanol did not exceed 0.1%. JMV-180 was initially prepared as a 200 μM stock solution in Me$_2$SO and then diluted in the extracellular solution; the concentration of Me$_2$SO did not exceeded 0.1%. During experiments, solutions were exchanged by perfusion using a gravity-fed system.

The intracellular solution (solution for loading into patch pipettes) contained 130 mM KCl, 10 mM NaCl, 1.5 mM MgCl$_2$, 2 mM ATP, 0.1 mM EGTA, and 10 mM HEPES (adjusted to pH 7.2 by KOH). 10 mM BAPTA and 2 mM CaCl$_2$ were added to the intracellular solution to obtain an intrapipette solution with highly buffered calcium.

Loading of Fluorescent Indicators and Optical Imaging of Cells—For the low concentration mode of measurement, cells were loaded with 50–100 nM TMRM (or TMRE) for 20–25 min at 37 °C. For the dequench mode of measurement, cells were loaded with 10–20 μM TMRM (or TMRE) for 20–25 min at 37 °C. TMRM and TMRE were initially prepared as 10 mM stock solutions in Me$_2$SO and diluted to the required concentration before loading into the cells. In the majority of experiments, fluorescence imaging of cells was conducted using a Leica SP2 confocal microscope with a × 63 water immersion objective. Fluorescence was excited by a 543-nm laser line, and emission was collected above 560 nm. In some experiments, a Zeiss confocal microscope LSM 510 was used with similar excitation/emission conditions.

During processing of fluorescence data, generated in experiments with low concentrations of TMRM/TMRE, the signals from mitochondria and cytosol were analyzed separately (*e.g.* see Fig. 1*B*). The regions of interest were chosen around perigranular mitochondria (the largest group of mitochondria in the cell) and in the cytoplasm devoid of mitochondria. As expected from the low concentration mode of measurements, the depolarization of mitochondria resulted in a decrease in mitochondrial fluorescence and an increase of fluorescence in the mitochondria-free parts of the cell. The fluorescence changes were similar in basal and apical nonmitochondrial regions; however, it was usually more convenient to analyze basal regions.

For analyses of the dequench mode experiments, regions of interest included whole cells. We have chosen this form of analysis, because both the mitochondrial region and the cytosol responded to depolarization of mitochondria with an increase of fluorescence. The increase of fluorescence in the mitochondrial region is probably due to the fact that the fluorescence increases in regions of the cytosol located between mitochondria in the mitochondrial belt more than compensate for the decrease of fluorescence in mitochondria.

One potential artifact in employing charged membrane-permeant indicators for measurements of $\Delta\psi_m$ is that the fluorescence can be influenced by translocation of the indicators across the plasma membrane. Because of the presence of calcium-dependent ionic channels in the plasma membrane, the pancreatic acinar cells are able to generate rapid changes of membrane potential during calcium responses (10). To avoid a possible artifact due to the redistribution of indicators across the plasma membrane, we removed the indicators from the extracellular solution prior to experiments. A few control experiments conducted in the continuous presence of TMRM in the extracellular solution are mentioned under "Effects of Physiological and Supramaximal Doses of CCK, Measured in Dequench Mode."

The fluorescence was usually corrected for bleaching, using the initial parts of the curves. Intracellular calcium was measured with fluo-4 on the Zeiss confocal microscope. In these experiments, 3 μM fluo-4, AM (membrane permeable form of the indicator; AM stands for acetoxymethyl ester) were loaded into cells for 20–25 min at room temperature. Fluo-4 was excited by a 488-nm laser line, and emission was collected through a 505–550-nm band pass filter.

Patch Clamp Recording—The electrophysiological recordings of calcium-dependent Cl$^-$ currents were used as a measure of changes in cytosolic calcium. Whole cell current recordings were made using an EPC-8 amplifier and PULSE software (HEKA). The holding potential was −30 mV. The pipette resistance usually was 2–3 megaohms.

Chemicals—TMRM, TMRE, and fluo-4, AM were purchased from Molecular Probes, Inc. (Eugene, OR). Bombesin, dibutyryl cyclic AMP, taurolithocholic acid 3-sulfate (TLC-S), taurodeoxycholic acid (TDC), taurochenodeoxycholic acid (TCDC), taurocholic acid (TC), and other chemicals were purchased from Sigma and were of the highest grade available. JMV-180 was purchased from Research Plus, Inc. (Bayonne, NJ).

FIG. 1. **Responses of the mitochondria membrane potential probe TMRM to mitochondrial inhibitors.** Comparison of low concentration and dequench mode of measurements. *A*, the sequential addition of increasing concentrations of CCCP to pancreatic acinar cells loaded with 10 μM TMRM (dequench mode). The trace shows normalized changes of fluorescence. *F* represents the intensity of fluorescence; F_0, the intensity of fluorescence at the beginning of an experiment. *B* (*a*), the sequential addition of increasing concentrations of CCCP to pancreatic acinar cells loaded with 100 nM TMRM (low concentration mode). The *upper trace* shows the fluorescence recorded from the perigranular mitochondrial region (of the *upper right cell* shown in *B* (*b*)). The *lower trace* represents fluorescence recorded from the basal nonmitochondrial region of the cytosol (of the same cell). *B* (*b*), images of a doublet of pancreatic acinar cells; the *upper part* shows the transmitted image of the cells, and the *lower part* represents the confocal image of TMRM distribution (*i.e.* distribution of mitochondria; note the low density of mitochondria in the basal regions). The *bar* on the confocal image corresponds to 4 μm. *C*, the effect of rotenone/oligomycin (*R/O*; 2 μM/2 μM) followed by 10 μM CCCP on mitochondrial membrane potential in pancreatic acinar cells loaded with 10 μM TMRM (dequench mode).
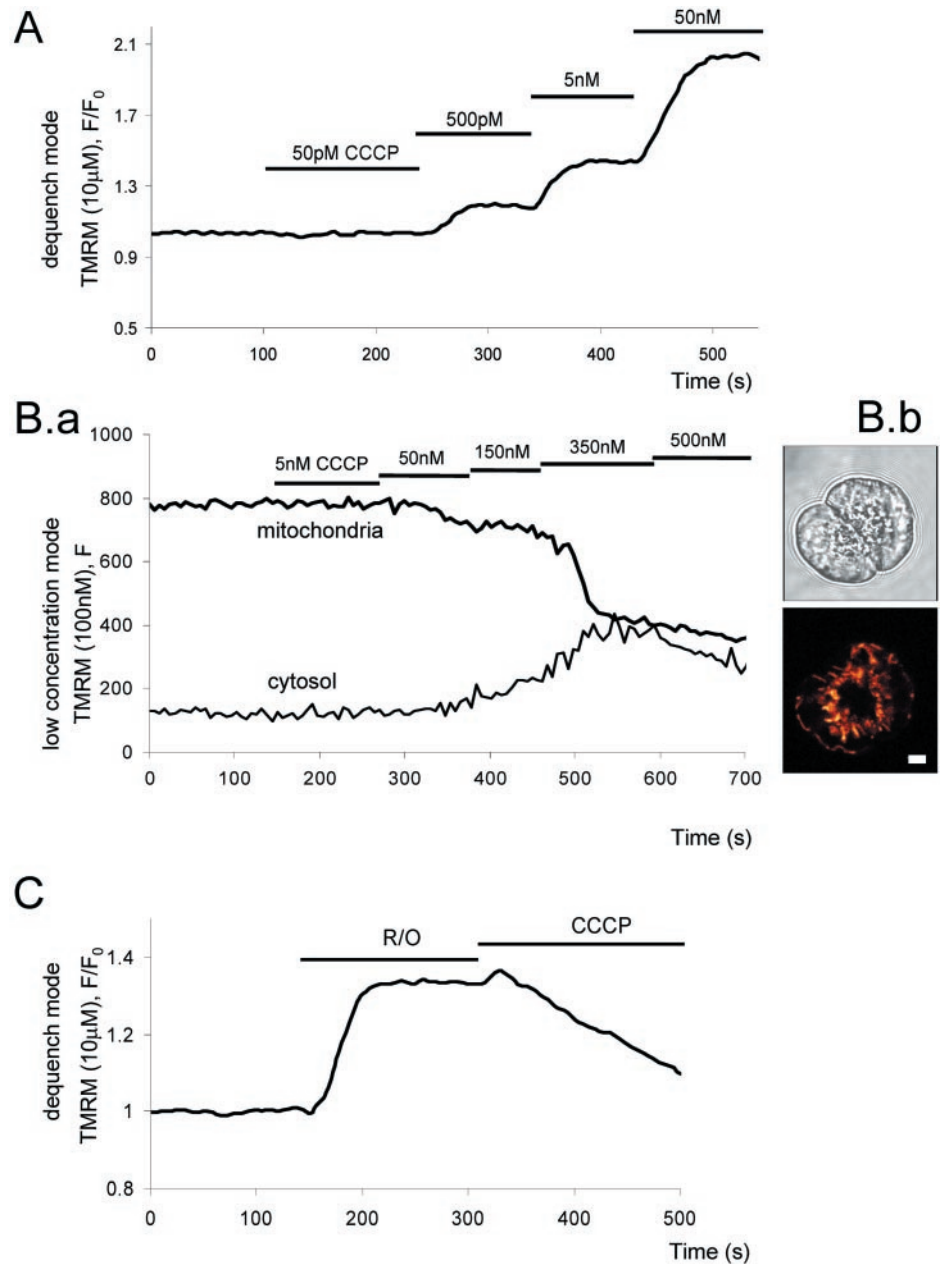
RESULTS

Comparison of the Sensitivity of the Dequench Mode and the Low Concentration Mode of Mitochondrial Membrane Potential Measurements

Fig. 1 shows results of experiments designed to reveal the difference in sensitivity of the two modes of measurements of the mitochondrial membrane potential. Fig. 1*A* represents the dequench mode of measurements, whereas Fig. 1*B* illustrates results obtained with low concentration loading of TMRM.

Application of the protonophore CCCP (Fig. 1*A*) resulted in increases of fluorescence in cells loaded with a high concentration (10 μM) of TMRM ($n = 16$). The threshold CCCP concentration, able to produce a response, was between 50 and 500 pM. No responses were found at 5 pM CCCP ($n = 6$; not shown). The majority of cells did not respond to 50 pM CCCP (11 of 16 cells, with small responses in five cells), whereas all cells ($n = 16$) responded to 500 pM CCCP. The subsequent addition of 5 and 50 nM CCCP resulted in further increases of fluorescence in all cells (Fig. 1*A*). During CCCP-induced responses, the fluores-

cence increased in all regions of the cells (not shown). These results are consistent with the dequench mode of measurements. Single application of high doses of CCCP (500 nM or higher) resulted in a fast and large increase of fluorescence followed by a slower decline, due to loss of the indicator from the cells (see examples in Figs. 2–7). Such additions of the protonophore were used at the end of the experiments to reveal the presence of dequenched indicator in the mitochondria. In separate experiments, we determined the amplitude of the fluorescence rise induced by application of a very high concentration of CCCP (10 μM). In these experiments, the fluorescence increased by 82 $\pm$ 8% ($n = 31$). Here, and below, the values represent averaged changes of fluorescence divided by the fluorescence value taken just before the addition of the relevant substance and multiplied by 100% $\pm$ S.E. of such measurements.

The low concentration mode of TMRM measurements was much less sensitive (Fig. 1*B*, *a*). In cells loaded with 100 nM TMRM, low concentrations of CCCP (50 and 500 pM) did not produce measurable changes of fluorescence ($n = 6$), whereas 5
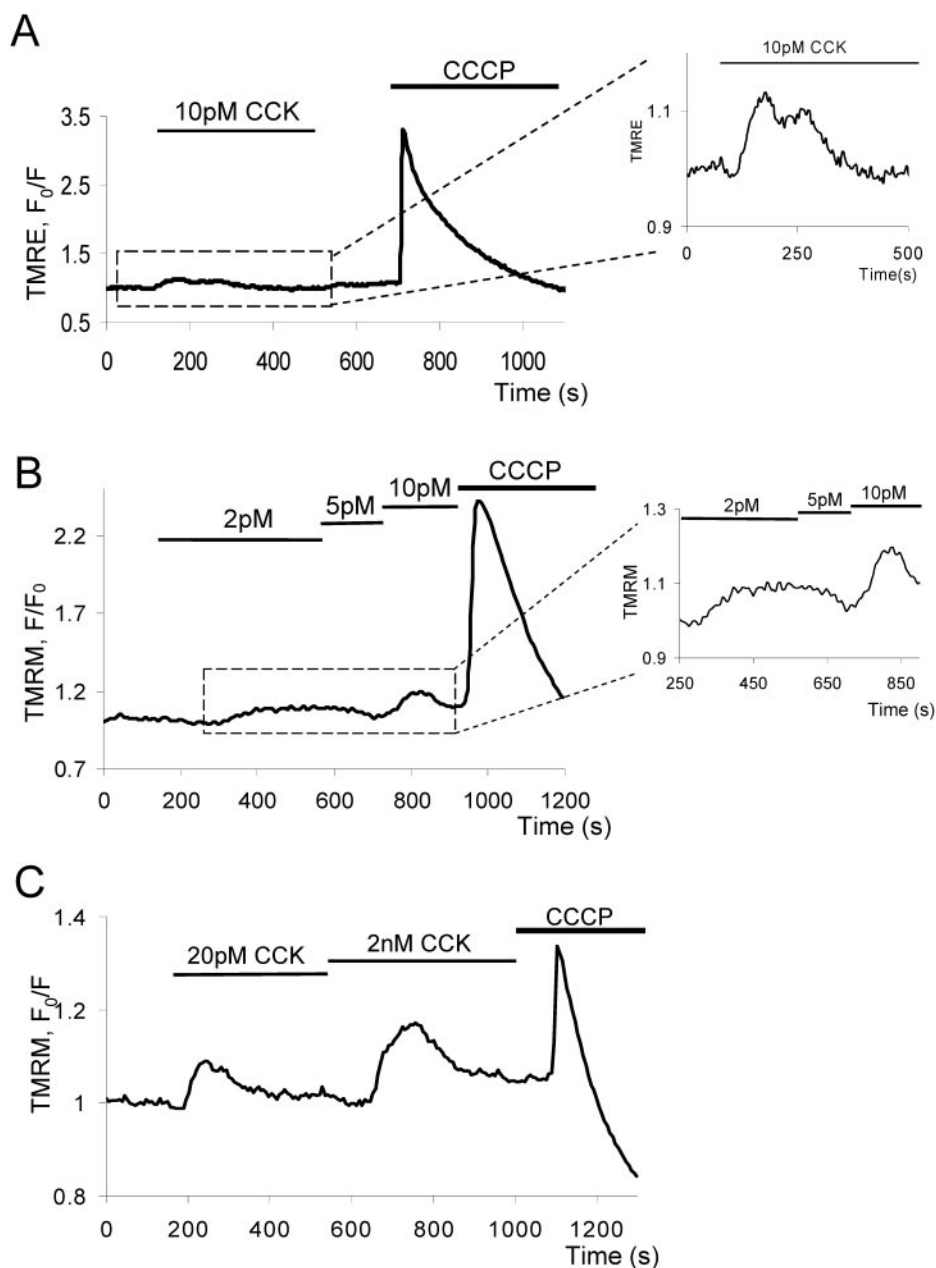
FIG. 2. **Changes of mitochondria membrane potential induced by CCK.** Measurements of the mitochondria membrane potential were performed using the dequench mode of the fluorescence probes TMRM or TMRE. A mitochondrial uncoupler CCCP (10 μM) was added at the end of the experiments. *A*, normalized fluorescence changes induced by 10 pM CCK. Expanded time and fluorescence scales are used in the *inset* to highlight the CCK effect. *B*, the effect of the sequential addition of 2, 5, and 10 pM CCK. *C*, normalized fluorescence changes induced by the addition of 20 pM and 2 nM CCK.

nM produced responses in a minority of cells (3 of 30 cells). At 50 nM CCCP, the proportion of responding cells increased to ~30% (11 of 34 cells; Fig. 1*B*, *a*). With the sequential addition of increasing concentrations of CCCP, all cells responded before the concentration of the protonophore reached 500 nM (usually when the concentration reached 150–350 nM). Because of clustering of mitochondria in the apical region and the fact that the basal region has a very low density of mitochondria (see images of the cells in Fig. 1*B*, *b*), we were able to measure the redistribution of TMRM (and TMRE) from mitochondria to mitochondria-free regions of the cytosol. At concentrations from 50 to 150 nM, CCCP produced a slow decrease in the mitochondrial fluorescence, and this was (as expected) accompanied by a slow fluorescence increase in the nonmitochondrial regions of the cytosol. The steepness of the fluorescence changes increased with increasing CCCP concentration (Fig. 1*B*, *a*). The threshold concentration of CCCP for the responses recorded in the low concentration mode was usually between 50 and 150 nM.

These results indicate that the dequench mode is more than 2 orders of magnitude more sensitive than the low concentration mode (in terms of the concentration of CCCP necessary to induce measurable fluorescence changes).

To further test whether the dequench mode reflects depolarization of mitochondria, we conducted experiments with application of the mitochondrial inhibitors rotenone and oligomycin. The application of rotenone/oligomycin (both substances at a concentration of 2 μM), resulted in a strong increase of TMRM fluorescence (Fig. 1*C*, $n = 15$). Further application of CCCP produced only a very small fluorescence rise followed by recovery. Similar results were obtained with application of rotenone (2 μM) without oligomycin (not shown; $n = 4$). The results presented here demonstrate that the dequench mode of measurements reflects depolarization of mitochondria and that this method of evaluation of $\Delta\psi_m$ is considerably more sensitive than the low concentration mode. Below, we describe the effects of calcium-releasing secretagogues and bile acids, examined using the dequench mode in cells loaded with TMRM or TMRE.

Effect of the Calcium-releasing Secretagogue CCK on Mitochondrial Membrane Potential

Effects of Physiological and Supramaximal Doses of CCK, Measured in Dequench Mode

In a previous study from our group (22), we used the low concentration mode of TMRE loading and found no changes of fluorescence even when cells were stimulated with high supramaximal concentrations of CCK. Having established that the dequench mode of TMRM (or TMRE) loading provides a much more sensitive method for elucidation of changes of Δψ$_m$, we decided to reexamine the effects of calcium-releasing agonists on the mitochondrial membrane potential. To our surprise, even small physiological doses of CCK produced measurable changes of TMRE and TMRM fluorescence. Fig. 2*A* shows a TMRE fluorescence record. Stimulation with 10 pM CCK (the concentration seen in the plasma after a meal) (39) resulted in an increase of TMRE fluorescence in 14 of 17 cells tested (82% of cells). Similar results were obtained with TMRM. In TMRM-loaded cells, 10 pM CCK induced a measurable increase of fluorescence in 19 of 25 cells (76% of cells). The responses to this upper range physiological dose of CCK were composed of a single slowly recovering transient (or infrequently two transients). Neither of the indicators showed oscillations of fluorescence. At the end of each experiment, we applied a high concentration of CCCP (10 μM) to verify the presence of the operational dequench mode; this resulted in a fast rise of fluorescence followed by a slower recovery (Fig. 2*A*). A low CCK concentration (2 pM), only slightly higher than the concentration found in serum of fasting animals (39), was able to induce responses in 12 of 36 cells (30% of cells) examined using TMRM loading (see Fig. 2*B*). The fluorescence changes induced by 2 pM CCK varied considerably from an absence of responses to delayed slowly developing responses and fast responses. Subsequent sequential applications of 5 and 10 pM CCK produced an additional fluorescence increase to one or both of these concentrations. In experiments with sequential stimulation with rising concentrations of CCK, the majority of the cells that had not responded to 2 pM eventually responded to either 5 or 10 pM CCK. Stimulation of cells with 2 nM CCK (high, supramaximal concentration) resulted in clear dequench responses in the majority of cells loaded with TMRM (8 of 11 cells) or TMRE (26 of 32 cells) (not shown). In experiments in which mitochondria were first depolarized by 10 or 20 pM CCK and then exposed to a higher concentration (2 nM) of the secretagogue, the higher dose of CCK almost always produced additional mitochondrial depolarization (18 of 21 cells; Fig. 2*C*).

In the experiments described above, we removed the mitochondrial indicators from the extracellular solution. Therefore, an influx of indicators could not contribute to the rise of fluorescence.

We also conducted a number of dequench mode experiments (*n* = 16) in the continuous presence of TMRM (50 nM to 2 μM) in the extracellular solution. In this condition, CCK (20 or 50 nM) also produced increases of TMRM fluorescence.

Since the extracellular indicator-free mode of dequench measurements provides a more stringent test for mitochondrial depolarization, all further experiments were conducted in this configuration.

Calcium Dependence of the CCK Effect on Mitochondrial Potential

CCK is a calcium-releasing secretagogue. It has been suggested that calcium entry into mitochondria could result in mitochondrial depolarization (27). It is therefore reasonable to hypothesize that the CCK effect, observed in our experiments, is mediated by mitochondrial calcium entry.

To reveal the calcium-dependent component of the CCK-induced fluorescent changes, we conducted experiments at high or low cytosolic calcium buffering. After loading with high concentrations of TMRM, cells were patched (whole cell configuration) and stimulated with CCK. Fig. 3*A* shows the result of a representative experiment (*n* = 5) in which the patch pipette (and consequently the cytosol of the cell) contained a low concentration of the calcium chelator EGTA (100 μM). In this case, CCK stimulations induced the expected increases of TMRM fluorescence. The *upper trace* of the figure shows the calcium-dependent Cl$^-$ current, which reflects cytosolic calcium concentration changes; the *lower part* represents TMRM responses.

Our ability to resolve TMRM responses in voltage clamp conditions strongly indicates that the dequench responses reflect depolarization of mitochondria and not redistribution of the indicator across the plasma membrane.

The CCK-induced calcium-dependent current was completely blocked (see Fig. 3*B*) when the patch pipette solution contained a high concentration of calcium buffer (10 mM BAPTA, 2 mM CaCl$_2$). In this case, CCK-induced TMRM responses were also completely inhibited (see Fig. 3*B*; *n* = 4).

These experiments strongly suggest that the CCK-induced changes of mitochondrial membrane potential occurred as a consequence of the cytosolic calcium rise.

To verify that the calcium rise is itself sufficient to induce an increase of TMRM fluorescence, we conducted experiments in which the calcium release from internal stores was triggered in an agonist-independent manner. The SERCA pump inhibitor thapsigargin is known to release calcium from internal stores of pancreatic acinar cells and increase the cytosolic calcium concentration (40–42). Application of thapsigargin to cells loaded by a high concentration of TMRM (dequench mode) resulted in an increase of fluorescence (in 21 of 29 cells; see Fig. 3*C*). These results are consistent with calcium-dependent depolarization of the mitochondria.

Confirmation of Absence of CCK Effects in Measurements Utilizing Low Concentration Mode

The results of CCK stimulation, obtained using the dequench mode of TMRM/TMRE, contradict the published outcome of experiments conducted using the low concentration mode, where no responses to CCK or ACh application were found (22, 43). We therefore decided to repeat the low concentration mode measurements of the CCK effects. We used the low concentration (100 nM) loading protocol for TMRM and tested the mitochondrial fluorescence responses to different doses of CCK (10 pM, 1 nM, 2 nM, and 10 nM). We confirmed our previous finding. Indeed, the majority of the cells loaded with low doses of TMRM did not show any fluorescence changes when challenged by these doses of CCK (not shown). Only 2 of 21 cells showed a small (less than 10%) decrease of fluorescence in mitochondrial regions when stimulated with 10 pM CCK. The fluorescence of other cells did not change during such CCK applications. Higher doses of CCK were also usually without effect (only 2 of 21 cells responded to 1 nM of CCK, zero of seven cells responded to 2 nM of CCK, and only 1 of 14 cells responded to 10 nM CCK). The drastic differences in the sensitivity of the dequench and the low concentration modes, described under "Comparison of the Sensitivity of the Dequench Mode and the Low Concentration Mode of Mitochondrial Membrane Potential Measurements," were clearly evident also in the experiments with CCK stimulation.

Effects of Other Calcium-releasing Agonists JMV-180 and Bombesin

Both bombesin and JMV-180 produced measurable (in the dequench mode) changes of TMRM fluorescence. The responses
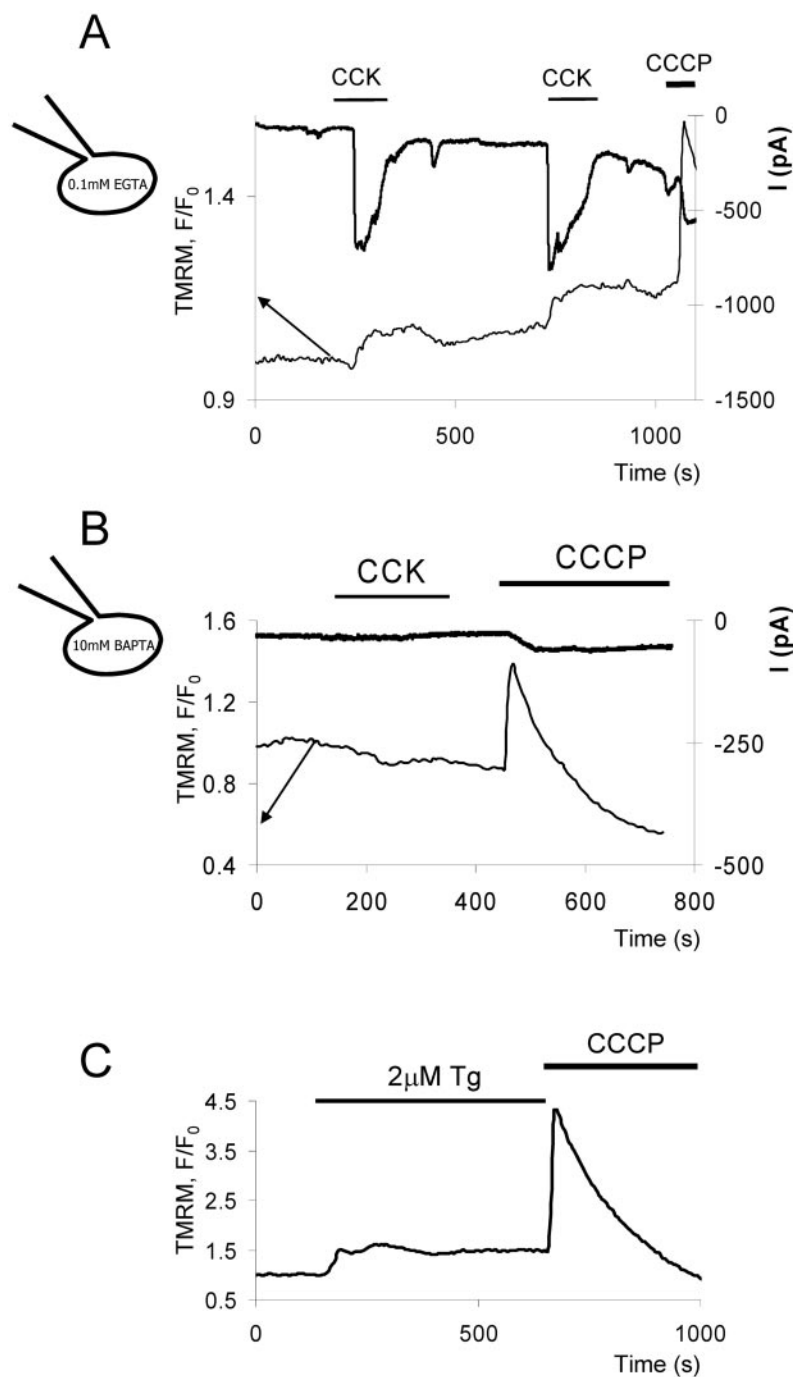
FIG. 3. **Calcium dependence of CCK-induced mitochondrial depolarization and mitochondrial depolarization triggered by thapsigargin.** Simultaneous recordings of mitochondrial membrane potential and cytosolic calcium responses induced by 2 nM CCK (*A* and *B*). Whole cell current recordings of calcium-dependent chloride currents were used to monitor cytosolic calcium changes. The holding potential was −30 mV. The *upper traces* for both *A* and *B* show recordings of calcium-dependent current, and the *lower traces* show changes of TMRM fluorescence (in both *panels*, an *arrow* links a fluorescence trace with an appropriate axis). *A*, calcium-dependent Cl⁻ current (*upper trace*) and TMRM fluorescence (*lower trace*) in a patch-clamped cell with low calcium buffering (0.1 mM EGTA in the patch pipette solution). *B*, inhibition of calcium current and mitochondrial depolarization by high calcium buffering (10 mM of BAPTA, 2 mM CaCl₂ in the patch pipette). *C*, changes of TMRM fluorescence in intact (nonpatched) pancreatic acinar cell induced by 2 μM thapsigargin (*Tg*).

to bombesin (Fig. 4) were qualitatively similar to those found for CCK. Low concentrations of bombesin (5 pM) produced a small depolarization of mitochondria (in 9 of 28 cells). All cells responded to 10 pM (*n* = 3) and 50 pM (*n* = 3) bombesin. In cells responding to 50 pM, subsequent application of 5 nM of bombesin triggered a further, more substantial fluorescence increase (*n* = 3).

TMRM responses to high concentrations of JMV-180 (20–100 nM) were small and slowly developing. Following responses to either 20 or 100 nM JMV-180, application of 10 nM CCK produced an additional increase of TMRM fluorescence (eight of eight cells; Fig. 4*B*). In separate experiments, we recorded (using fluo-4) calcium responses to 20 and 100 nM JMV-180. Calcium responses to 20 and 100 nM JMV-180 were composed of periodical base-line transients in all tested cells

(14 cells; not shown). In these experiments, we have not recorded sustained calcium elevations.

Secretin Does Not Produce Mitochondrial Depolarization

Secretin is the secretagogue that in pancreatic acinar cells operates primarily through the cAMP pathway (36). Secretin, tested at concentrations of 30 pM, 1 nM, and 100 nM, did not produce resolvable changes of TMRM fluorescence in the dequench mode of measurement (Fig. 5*B*; *n* = 19, 19, and 26 correspondingly). Dibutyryl cyclic AMP (0.1 mM) was also without effect (Fig. 5*A*; *n* = 23).

In experiments with fluo-4, we found that secretin at concentrations of 30 pM (*n* = 11) and 1 nM (*n* = 11) failed to trigger calcium responses in all tested cells. At a higher concentration (100 nM), secretin induced calcium oscillations in a small pro-
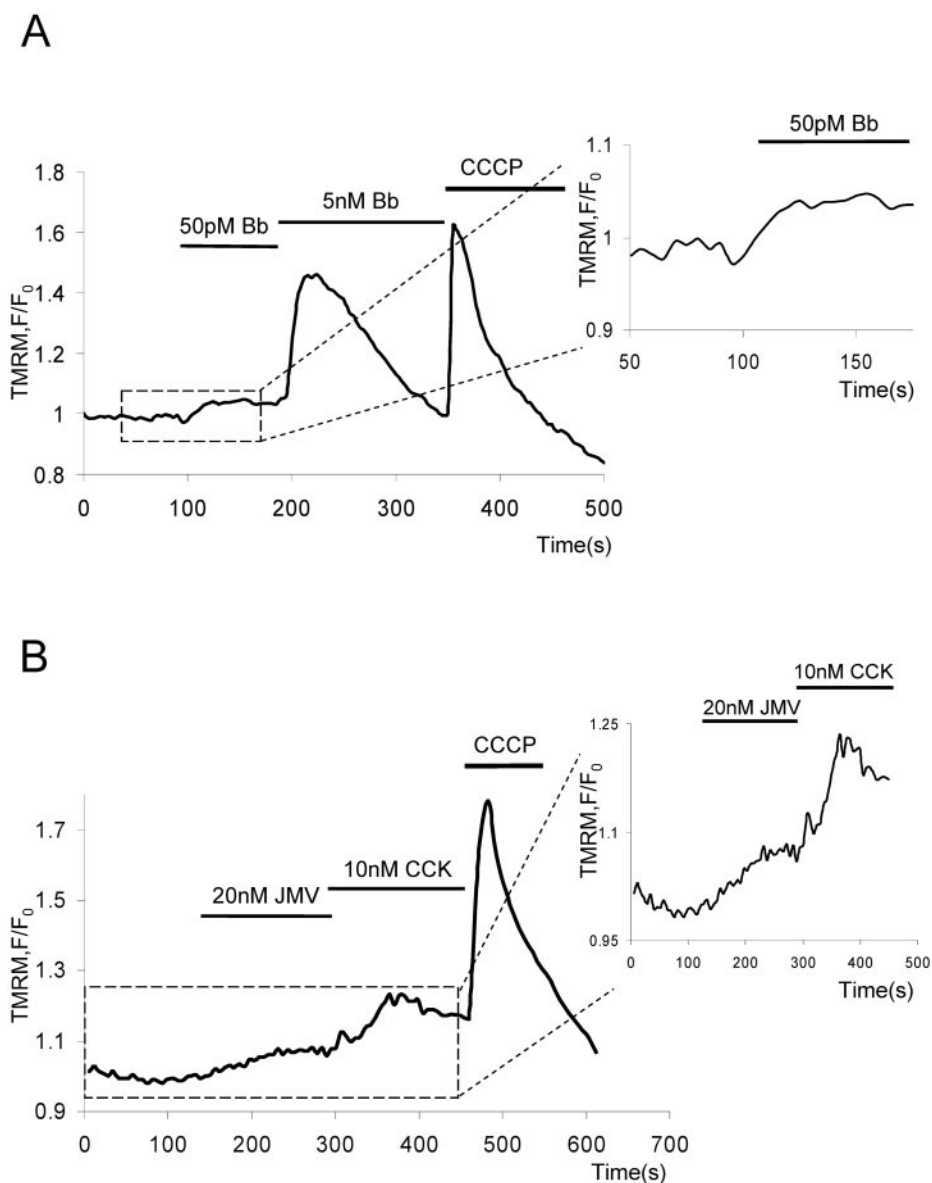
FIG. 4. **Depolarization of mitochondrial membrane potential induced by the calcium-releasing agonists bombesin and JMV-180.** Evaluation of changes in mitochondrial membrane potential was performed on cells loaded with 10 μM TMRM. *A*, sequential application of 50 pM, 5 nM bombesin, and 10 μM CCCP stimulated measurable elevation of TMRM fluorescence. An *inset* shows the effect of 50 pM bombesin on expanded scale. *B*, stimulation of mitochondrial depolarization by 20 nM JMV-180 and 10 μM CCK. The subsequent application of 10 mM CCCP produced further depolarization. Expanded time and fluorescence scales are used in the *inset* to highlight the effect of JMV-180 and CCK.

portion of the cells (3 of 24 cells). Sustained calcium elevations were not recorded for any concentration of secretin.

Effect of Bile Acids on Mitochondrial Membrane Potential

Effects of TLC-S Measured Using Dequench and Low Concentration Modes

Dequench Mode—Fig. 6 shows that the bile acid TLC-S induced fluorescence increases in cells stained with the high concentration (dequench) mode of TMRM, but not in cells stained with the low concentration of TMRM.

A very small concentration of this bile acid (10 μM) was able to induce resolvable changes in fluorescence (in 10 of 15 cells), when measured using the dequench mode (see Fig. 6*A*). A subsequent increase of the TLC-S concentration to 25 μM resulted in a further increase of TMRM fluorescence. TLC-S (25 μM) produced depolarization of mitochondria in 21 of 23 cells. We also characterized the responses to 50, 100, and 200 μM TLC-S; the proportion of cells that responded to these concentrations were 20 of 22, five of five, and three of three, respectively (see also Fig. 6*B*). In patch clamp experiments, buffering of the intrapipette (and consequently the intracellular) solution with 10 mM BAPTA and 2 mM CaCl$_2$ blocked the mitochondrial depolarization induced by 10 and 25 μM TLC-S but did not

abolish the mitochondrial depolarization induced by 100 μM TLC-S ($n = 4$; Fig. 6*C*). However, in these experiments, substantial further dequench was induced by CCCP. These experiments reveal a calcium-independent component of the mitochondrial response to higher doses of TLC-S.

Low Concentration Mode—The measurements with low concentration of TMRM were much less sensitive (Fig. 6*C*); no responses were recorded for 200 μM TLC-S ($n = 12$). The majority of cells did not respond to 400 μM TLC-S (10 of 12 cells; Fig. 6*D*); even at 600 μM, TLC-S induced responses in only three of nine cells. Note that the lowest concentration that was used in this mode of measurement (and completely ineffective) was the same as the highest concentration used for the dequench mode (and effective in all tested cells).

Effects of Bile Acids TC, TDC, and TCDC Characterized in Dequench Mode

In our previous work, we characterized calcium signaling induced by bile acids. Among the tested bile acids, TLC-S was by far the most potent calcium releaser. TDC was less potent, and TC was the least effective (44). In our current study, we used the dequench mode of TMRM loading to investigate the effects of TC, TDC, and TCDC on mitochondrial membrane
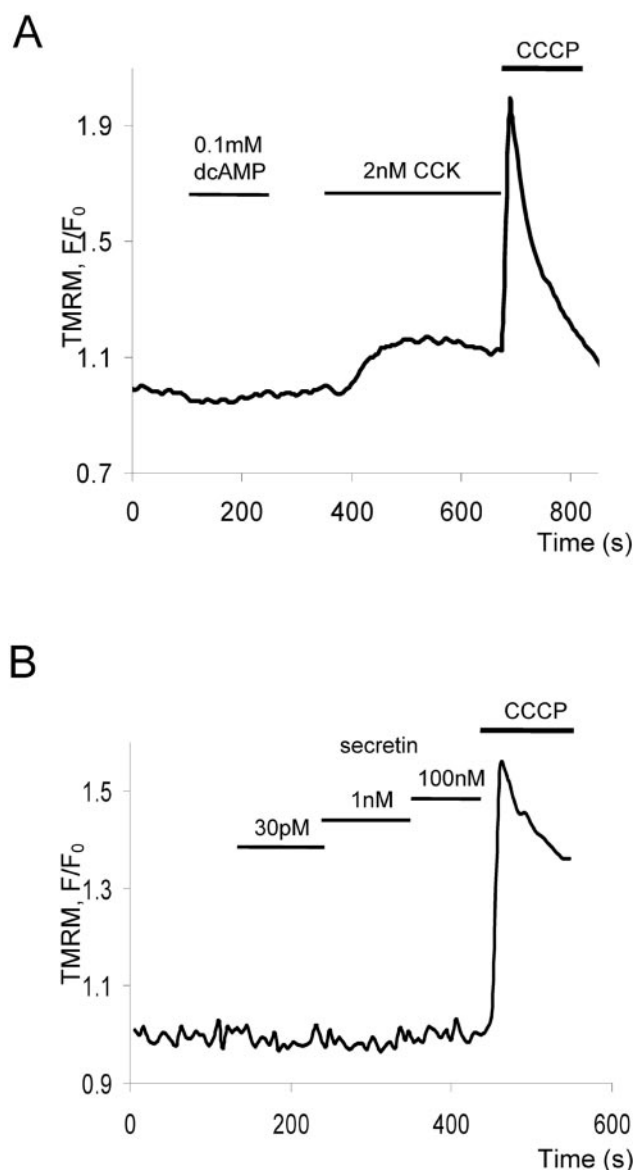
Measurements of $\Delta\psi_m$ in Pancreatic Acinar Cells

A

B

FIG. 5. **The lack of changes of mitochondrial membrane potential on application of dibutyryl cyclic AMP (*dcAMP*) and secretin.** *A*, an example of response from the cell loaded with 10 μM TMRM on subsequent application of 0.1 mM dibutyryl cyclic AMP, 2 nM CCK, and 10 μM CCCP. *B*, the lack of changes in fluorescence on application of 30 pM, 1 nM, and 100 nM of secretin, followed by a strong increase of TMRM fluorescence upon the addition of CCCP.

potential. Fig. 7 shows changes of TMRM fluorescence induced by TC, TDC, and TCDC.

In a majority of cells, 5 mM TC produced no measurable changes of TMRM fluorescence (Fig. 7*A*, *a*). Only in 4 of 16 cells did TC application produce increases of fluorescence (Fig. 7*A*, *b*).

TDC at concentrations of 1 mM and 100 μM produced mitochondrial depolarization in all cells tested (*n* = 9; Fig. 7*B*). At the lower concentration of 10 μM, TDC produced responses in only two of eight cells (Fig. 7*B*, *b*). Subsequent application of 100 μM TDC produced a rise in fluorescence in all of these cells (Fig. 7*B*, *b*). We also characterized the calcium responses to 10 μM and 100 μM TDC, since this was not done in our previous study. At 10 μM, this bile acid produced small local calcium signals in 5 of 13 cells. The higher concentration of TDC (100 μM) induced preferentially apical transients in all cells tested (*n* = 16). Subsequent application of 1 mM of TDC produced large global calcium transients (not shown).

Another bile acid, TCDC, produced mitochondrial depolarization in 19 of 37 cells, when applied at 1 mM (Fig. 7*C*, *a*). No responses were induced by a 10 μM concentration of this bile acid (*n* = 10), but intermediate concentrations of TCDC (100, 300, and 600 μM) produced responses in some of the cells tested (Fig. 7*C*, *b*). Half of the cells tested responded to 600 μM TCDC (12 of 24 cells). The proportion of responding cells decreased to 24% for 100 μM (*n* = 37).

We also characterized calcium responses induced by TCDC, since this was not done in our previous study. Application of 10 μM TCDC produced no measurable calcium changes (*n* = 3). Local apical calcium transients were induced by 100 μM TCDC in two of five cells tested (three cells did not show any calcium changes). TCDC (1 mM) triggered global calcium oscillations in five of five cells tested; in 3 of these 5 cells the oscillations were superimposed on an elevated calcium plateau (not shown).

DISCUSSION

Our previous study of stimulus-metabolism coupling in pancreatic acinar cells (32) revealed both positive and negative changes of NADH concentration occurring in mitochondria of pancreatic acinar cells due to action of calcium releasing secretagogues. Whereas positive deflections on traces of NADH fluorescence are best explained by calcium-dependent activation of dehydrogenases of the Krebs cycle, the decreases are consistent with depolarization of mitochondria due to Ca^{2+} entry, via the mitochondrial uniporter (27). Prior to this study, we had not seen these expected agonist-induced mitochondrial depolarizations. Previous measurements of $\Delta\psi_m$ in our laboratory where performed using low concentrations of rhodamine-based fluorescent indicators. This method of measurement is usable in some experimental conditions. Transient mitochondrial depolarizations induced by menadione were, for example, clearly resolved (43). However, no mitochondrial depolarizations were recorded in cells stimulated by CCK or ACh (22, 43) (these experiments were conducted both in the presence and in the absence of indicators in extracellular solution). The apparent discrepancy between our previous findings could be attributed to the insufficient sensitivity of the low concentration mode of mitochondrial measurements. We therefore attempted to test the alternative high concentration "dequench" mode of measurements. The dequench mode of measurement of $\Delta\psi_m$ was characterized in a number of studies (for an overview of methods of $\Delta\psi_m$ measurements, see Ref. 33) but has never been used in pancreatic acinar cells. We therefore started by directly comparing the relative sensitivity of the two modes of measurements. Surprisingly, such a comparative study has not been done before for any cell type. We found that the sensitivity of the dequench mode is substantially higher (more than 2 orders of magnitude) than that of the low concentration mode. In calibration experiments with different doses of CCCP, we were able to record remarkably reproducible, graded responses to very small concentrations of the protonophore. The effect of the protonophore could be potentially complicated due to its effect on pH gradients of other cellular organelles. We therefore conducted experiments with the mitochondrial inhibitor rotenone. The ability of rotenone (alone or in combination with oligomycin) to trigger complete dequenching of TMRM strongly indicates that the indicator is indeed dequenched due to its release from mitochondria. It has been reported that at high concentrations, fluorescent lipophilic cations inhibit mitochondrial respiration (45). For concentrations similar to those used in this study, the inhibitory effect of TMRM (the main indicator used in our experiments) on respiration of cardiac mitochondria was ~20% (45). This is a significant inhibition but not a major inhibition. The TMRM effect on respiration was less pronounced than that for TMRE (used in a few of our experi-
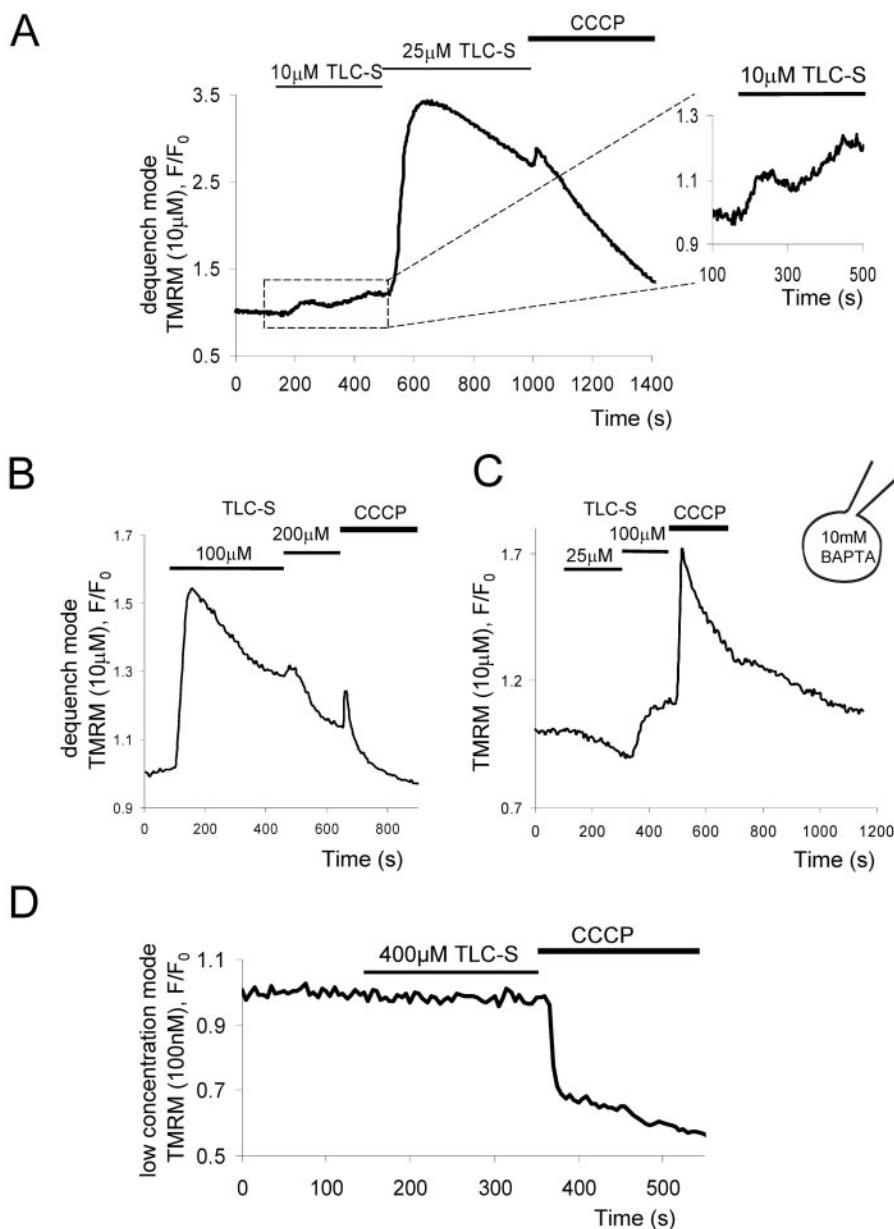
FIG. 6. **Depolarization of mitochondrial membrane potential induced by TLC-S.** *A*, changes of fluorescence produced by sequential application of 10 and 25 μM TLC-S and 10 μM CCCP in pancreatic acinar cells loaded with 10 μM TMRM (dequench mode). Expanded time and fluorescence scales are used in the *inset* to highlight the effect of 10 μM TLC-S. *B*, normalized fluorescence changes induced by 100 and 200 μM TLC-S, followed by 10 μM CCCP. *C*, Ca^{2+} chelator BAPTA suppressed responses to a low (25 μM) but not to a high (100 μM) concentration of TLC-S (dequench mode). *D*, the lack of changes in fluorescence upon application of 400 μM TLC-S to acinar cells, loaded with 100 nM TMRM (low concentration mode).

ments) and rhodamine 123. We cannot exclude the possibility that the rate of mitochondrial recovery and the absolute amplitude of $\Delta\psi_m$ are affected by TMRM, but our ability to resolve substantial increases of fluorescence, triggered by both calcium rises and by application of mitochondrial inhibitors, clearly indicates that the mitochondria are polarized, that the indicator is usable for these experiments, and that the procedure of measurements is highly sensitive to changes in $\Delta\psi_m$.

The high sensitivity of the dequench mode has been further confirmed by its capability to resolve mitochondrial effects of very small doses of the secretagogue CCK. It is remarkable that 30% of the cells responded to 2 pM CCK. The experience of our laboratory is that even calcium responses could not systematically be obtained at this secretagogue concentration. Surprisingly, the dequench mode seems to be a more sensitive detector of the presence of secretogue than cytosolic calcium measurements. One possible explanation for this could be that mitochondria are strategically positioned to detect even very small calcium releases when they occur from the endoplasmic reticulum. Preferential calcium uptake by mitochondria and close juxtaposition of mitochondria and endoplasmic reticulum have indeed been documented for other cell types (46–49). A recent

electron microscopy study from our laboratory, conducted on pancreatic acinar cells, clearly showed close positioning of ER and mitochondria and also a remarkable "contour following" of the membranes of these two organelles (50). This structural relationship probably allows mitochondria to import calcium during even minor release events from the ER and to be depolarized by this calcium entry. The finding that a low physiological range of CCK is capable of producing mitochondrial depolarization suggests that calcium-releasing secretagogues produce continued stimulating effects on mitochondria, by delivering calcium for efficient running of the Krebs cycle and other calcium-dependent mitochondrial functions. The calcium dependence of CCK-induced mitochondrial depolarization was confirmed by two lines of experiments. In combined confocal/patch clamp measurements, we demonstrated that strong buffering of cytosolic calcium completely prevented the CCK-induced rise of TMRM fluorescence. In experiments with thapsigargin, we demonstrated that the calcium rise produced in an agonist-independent fashion was indeed capable of causing mitochondrial depolarization. Taken together, these experiments strongly suggest that it is the calcium rise that is primarily responsible for mitochondrial depolarization and not

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FIG. 7. **Changes of mitochondrial membrane potential induced by TC, TDC, and TCDC.** Evaluation of changes in mitochondrial membrane potential were performed using the dequench mode of TMRM measurement. CCCP was applied at the end of each of the illustrated experiments to verify the presence of the dequenched indicator in the mitochondria. *A* (*a*), an example of a cell that did not change fluorescence upon application of 5 mM TC (representative of 12 of 16 cells). Note that subsequent application of 10 μM CCCP produced a clear and strong increase of TMRM fluorescence. *A* (*b*), an example of a cell that responded with an increase of fluorescence upon application of 5 mM TC (representative of 4 of 16 cells). B (*a*), an example of a fluorescence response induced by 1 mM TDC. *B* (*b*), fluorescence changes produced by the sequential addition of 10 and 100 μM TDC. *C* (*a*), an example of a fluorescence response produced by 1 mM TCDC. *C* (*b*), fluorescence changes induced by the sequential additions of 0.1, 0.3, 0.6, and 1 mM TCDC (periods of application of these increasing concentrations of TCDC are shown by *bars* in the *upper part* of this *panel*).
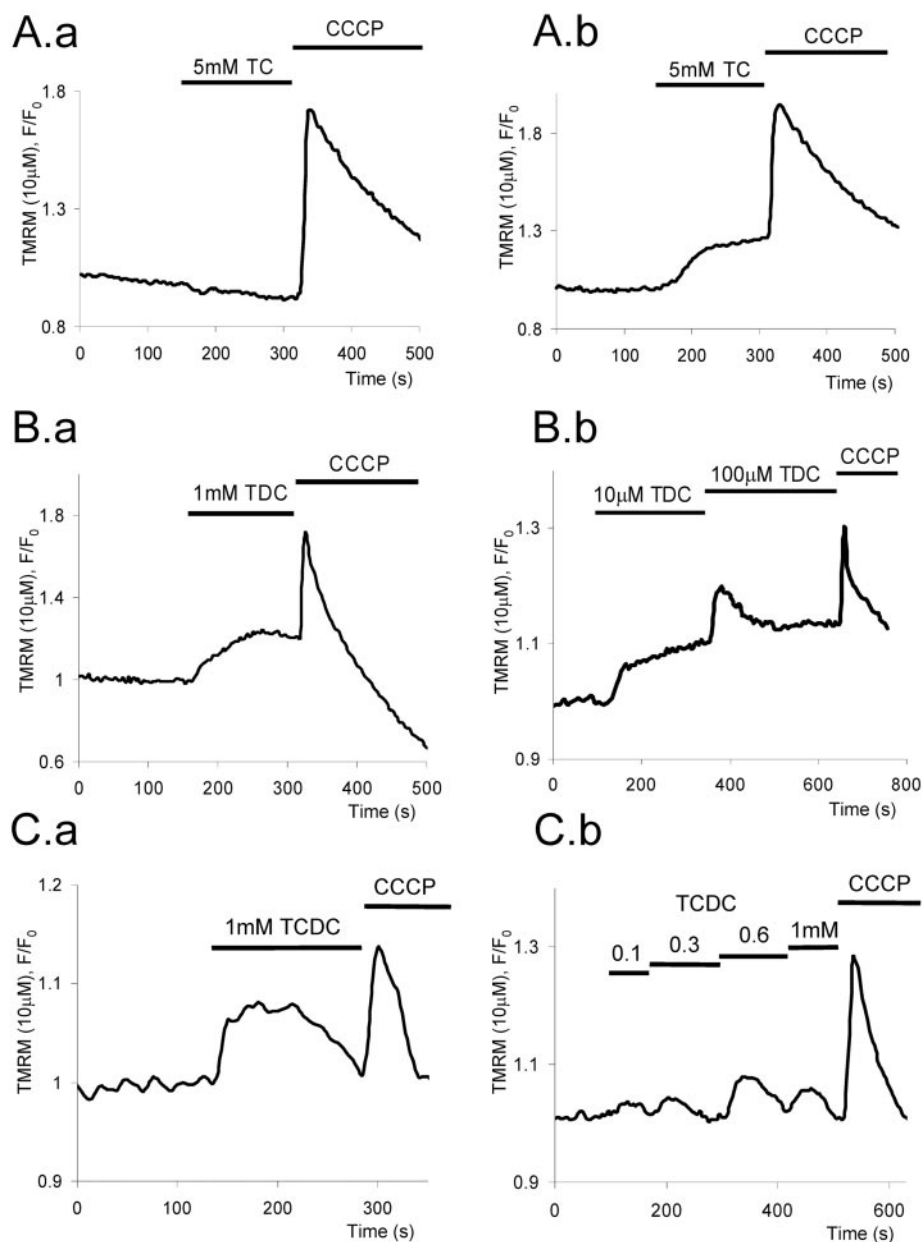
other signaling pathways activated by the secretagogue. The effects induced by low "physiological" concentrations of CCK on the cytosolic calcium concentration are highly variable, including global calcium transients, local apical calcium responses, or even absence of any calcium changes (10, 19, 51). It is therefore not surprising that the changes of $\Delta\psi_m$ are also variable, including relatively fast responses and slowly developing increases of fluorescence. In a substantial proportion of cells stimulated with 2 pM CCK, no changes of TMRM fluorescence were resolved. Starting from 10 pM (a concentration that corresponds to the upper limit of the physiological range), CCK produced $\Delta\psi_m$ responses in the majority of cells tested. Upon stimulation with 10 pM CCK, the fluorescence of TMRM-loaded cells increased by 9 ± 1% (n = 17, calculated for cells responding with a clearly defined CCK-induced fluorescence peak). This value is substantially smaller than the maximal fluorescence increase induced by a high dose (10 μM) of the protonophore CCCP (82 ± 8%), suggesting that CCK induced only a mild mitochondrial depolarization. Supramaximal CCK stimulation produced an additional depolarization of mitochondria. In experiments where stimulation with 20 pM CCK was fol-

lowed by stimulation with 2 nM CCK, the second stimulation always produced an additional increase of fluorescence. The fluorescence transient induced by 20 pM CCK was 9 ± 2%, and the subsequent fluorescence increase triggered by 2 nM CCK was 8 ± 3% (n = 8); both increases were substantially smaller than could be induced by complete depolarization of mitochondria with CCCP.

Physiological doses of CCK and low doses of ACh produce oscillatory calcium responses (18, 51, 52). In some of our experiments with small doses of secretagogues, we have seen a secondary rise of TMRM (or TMRE) fluorescence following the first transient, but we have never resolved oscillations of the fluorescence with recovery to base line, and most frequently, we only recorded single TMRM (TMRE) transients. In a number of control experiments conducted in the presence of TMRM in the extracellular solution, we also recorded more than one increase of fluorescence during CCK application, but in these conditions we were also unable to resolve oscillations. This probably reflects one of the disadvantages of the dequench mode of measurements. It seems that once an indicator is dequenched by a particular depolarization, it is

lost from the mitochondria and cannot be dequenched again by a depolarization of similar strength. A stronger depolarization, produced by a higher dose of agonist could trigger a subsequent response (Fig. 2, *B* and *C*) and further deplete the mitochondrial indicator. High doses of CCCP could usually trigger some further dequench of the probe. The restoration (requench) of the mitochondrial indicator in this cell type is most probably a slow process (in comparison with the period of CCK-induced oscillations). During stimulation, some indicator is undoubtedly lost from the cells; for strong and fast depolarizations with high concentrations of CCCP, the appearance of indicator could even be detected in the extracellular solution (when the confocal region of interest is placed in the vicinity of the cell membrane; not shown). It has been reported that the depolarization of the plasma membrane could speed up the loss of TMRM from the cytosol and prevent re-equilibration of indicator in the mitochondria (53). It is noteworthy that during stimulation with calcium-releasing agonists and with CCCP, the depolarization of the plasma membrane is expected to occur, due to activation of calcium-dependent currents (10). This could be an additional factor preventing reaccumulation of indicator in the mitochondria.

Despite these problems, the dequench mode of measurements resolved calcium-dependent depolarization of mitochondria by physiological and pathological doses of CCK.

Partial mitochondrial depolarization due to prolonged (0.5–3-h) treatment with CCK was reported previously (54). In this study, the physiological concentrations of the secretagogue were without effect, whereas supramaximal concentrations produced statistically significant mitochondrial depolarizations. The results are not necessarily contradictory to our study, since transient mitochondrial responses induced by low (physiological) doses of CCK (reported in our study) could be undetectable at later time points of measurements. The inhibition of mitochondrial function was also studied in a model of cerulein-induced acute pancreatitis. In these studies, the hyperstimulation with the CCK analogue, cerulean, produced considerable inhibition of mitochondrial respiration, decrease of the resting membrane potential, and decrease of the proportion of functioning mitochondria (55, 56). The measurements reported in these papers were done on a different time scale from our experiments. Taken together, the studies indicate that calcium-releasing secretagogues induce both a fast depolarizing effect and a prolonged depolarizing/damaging effect on the mitochondria of pancreatic acinar cells.

In our experiments, bombesin, which shows similar patterns of calcium signals to CCK, also caused qualitatively similar responses of TMRM fluorescence. This provides an additional argument for the notion that the mitochondrial depolarizations are triggered by calcium responses.

As expected from its calcium-releasing properties, JMV-180, even when applied at very high concentrations, produced only a moderate (the fluorescence change never exceeded 15%) and slow increase of TMRM fluorescence; subsequent stimulation with supramaximal concentration of CCK always produced an additional TMRM response.

It was interesting to test and compare the effects on $\Delta\psi_m$ of the secretagogues working through different second messenger cascades. Secretin is a pancreatic secretagogue that operates primarily through the cAMP signaling cascade. In our experiments, no resolvable changes in $\Delta\psi_m$ were produced by secretin. The membrane-permeable form of cAMP (dibutyryl cyclic AMP) was also ineffective. It is important to note that inositol 1,4,5-trisphosphate production and small calcium responses were reported for stimulation with high concentrations of secretin (57, 58). In our experiments, however, calcium responses

to secretin were much less pronounced than the responses induced by "classical" calcium-releasing secretagogues; furthermore, in our experiments, calcium responses to secretin were recorded only at a very high concentration and only in a few of the tested cells. The absence of $\Delta\psi_m$ changes during secretin applications is consistent with this finding.

The dequench mode of measurements was also shown to be more sensitive in resolving mitochondrial depolarization induced by bile acids. Bile acids can gain access to pancreatic acinar cells in certain pathological conditions and are considered as probable activators of acute pancreatitis (20, 59). The issue is far from resolved, since some studies question the role of bile in the pathology of acute pancreatitis (60, 61). However, bile acids have a drastic effect on the permeability of the pancreatic duct mucosal barrier (62), and two recent studies demonstrated that bile acids are capable of inducing sustained toxic calcium signals in pancreatic acinar cells (20, 44).

There is some discrepancy with regard to the suggested mechanism of bile-induced calcium elevation; whereas studies from our laboratory suggest potentiation of calcium release from the ER (44), work by J. Y. Kim and colleagues indicated inhibition of SERCA calcium pumps followed by activation of calcium influx (20). Nevertheless, both investigations clearly resolved major calcium changes and suggested that the action of bile acids is more specific than simply a detergent effect (20, 44).

We were able to resolve mitochondrial depolarizations produced by remarkably low concentrations of the bile acids TLC-S and TDC. As in the case with calcium releasing secretagogues, very low concentrations of bile acids produced mitochondrial depolarizations even more reliably than they triggered calcium signals. The effects of low concentrations of TLC-S were, however, completely abolished by BAPTA, suggesting that the effect of these low doses is calcium-mediated. Probably, the hypothesis of close connections between activated Ca^{2+} release sites and mitochondria could also be offered as explanation for such a high sensitivity of $\Delta\psi_m$ to bile acids. It is interesting to note that buffering of calcium was not efficient in blocking responses to higher concentrations of TLC-S. The doses of TLC-S that produce such depolarizations do not induce nonspecific loss of indicators from the cytosol (44) or from mitochondria (as evident from the low concentration mode of measurements). The action of TLC-S is therefore not due to its detergent effect. A more subtle ionophore effect of bile acids (63) could potentially be responsible for such an additional calcium-independent depolarization. TCDC is an important component of bile. We found that this bile acid was able to trigger calcium oscillations and mitochondrial depolarization starting from a concentration of 100 μM. Finally, TC was the least effective in inducing mitochondrial depolarization; even a relatively high concentration of this bile acid (5 mM) induced dequench of TMRM in a minority of cells. The mitochondrial effect of bile acids, found in this study, could be important for our understanding of the mechanism of cellular damage in bile-induced pancreatitis.

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