Fluctuations in mitochondrial membrane potential caused by repetitive gating of the permeability transition pore

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Confocal laser scanning microscopy and the potentiometric fluorescence probe tetramethylrhodamine ethyl ester were used to measure changes in membrane electrical potential ($\Delta\Psi_m$) in individual mitochondria after isolation or in the living cell. Recordings averaged over small mitochondrial populations revealed a gradual decline in $\Delta\Psi_m$ caused by the light-induced generation of free radicals. Depolarization was attenuated by dithiothreitol or acidification. In contrast, individual organelles displayed rapid spontaneous depolarizations caused by openings of the mitochondrial permeability transition pore (MTP). Repetitive openings and closings of the pore gave rise to marked fluctuations in $\Delta\Psi_m$ between the fully charged and completely depolarized state. Rapid spontaneous fluctuations in $\Delta\Psi_m$ were observed in mitochondria isolated from rat heart and in mitochondria in living endothelial cells. The loss of $\Delta\Psi_m$ of mitochondria in the living cell coincided with swelling of the organelle and the breakdown of long mitochondrial filaments. In the individual mitochondrion, oxidative stress initially triggered pore openings of shorter duration, before prolonged openings caused the complete dissipation of $\Delta\Psi_m$ and a measurable efflux of larger solutes. Generalizing this scheme, we suggest that under conditions of prolonged oxidative stress and/or cellular Ca^{2+} overload, short openings of MTP might serve as an emergency mechanism allowing the partial dissipation of $\Delta\Psi_m$, the fast release of accumulated Ca^{2+} ions and the decreased generation of endogenous oxygen radicals. In contrast, loss of matrix metabolites, swelling and other structural damage of the organelle render prolonged openings of the transition pore deleterious to mitochondria and to the cell.

Key words: free radical, mitochondrial compartmentation, tetramethylrhodamine ethyl ester.

INTRODUCTION

Mitochondrial Ca^{2+} overload in conjunction with oxidative stress can result in a sudden increase in the permeability of the inner membrane, causing the uncoupling of respiration from ADP phosphorylation. It is generally believed that this sudden increase in permeability is caused by the opening of a high-conductance pore residing in the inner mitochondrial membrane, i.e. the mitochondrial permeability transition pore (MTP) [1]. The pore permits the passage of solutes with a molecular mass of 1500 Da or less (reviewed in [2]). Both the molecular identity of the pore-forming structure and the detailed gating mechanism remain elusive. Current models suggest that the core structure of the pore is made up of a protein complex comprising the voltage-dependent anion channel of the outer membrane, the adenine nucleotide translocase of the inner membrane and the matrix space peptidyl prolyl-*cis*/*trans*-isomerase cyclophilin D. The three proteins form functional complexes located at contact sites between the inner and outer membranes and generate the basic behaviour of MTP when reconstituted into lipid bilayers [3] or liposomes [4]. However, the possibility cannot be excluded that the mitochondrial permeability transition is an epiphenomenon that might have different underlying mechanisms, all causing a marked increase in membrane permeability.

In a previous report [5] we demonstrated spontaneous fluctuations in electrical potential gradient ($\Delta\Psi_m$) in individual mitochondria isolated from cardiac muscle. Fluorescence signals recorded with the potentiometric dye tetramethylrhodamine ethyl ester (TMRE) showed that $\Delta\Psi_m$ oscillated between the fully charged and the completely depolarized state. Moreover, the rapid depolarizations coincided with an increase in permeability of the inner membrane for matrix-entrapped calcein. The results suggested that the light-induced generation of reactive oxygen species and the subsequent oxidation of protein thiols initiated the increase in permeability. Although individual organelles frequently displayed voltage fluctuations, $\Delta\Psi_m$ averaged over small mitochondrial populations (20–40 organelles) decayed gradually. The slope of the voltage decay was significantly decreased by cyclosporin A (CsA), a known inhibitor of MTP [2]. In addition, the radical scavenger catalase and GSH were found to be most effective in delaying MTP opening and mitochondrial depolarization. We therefore concluded that the light-induced depolarization was caused by opening(s) of MTP [5]. Here we provide further evidence that the rapid fluctuations in $\Delta\Psi_m$ observed in single mitochondria reflect openings of MTP. Voltage fluctuations in single isolated cardiac mitochondria were decreased by the thiol reductant dithiothreitol (DTT) and by acidification. Both experimental interventions are well known to inhibit MTP in suspensions of isolated organelles [2]. Detailed analysis of the kinetics of the voltage changes in single mitochondria revealed that short-lasting openings of MTP frequently preceded prolonged openings. Moreover, $\Delta\Psi_m$ of single mitochondria in living vascular endothelial cells was found to display fluctuations similar to those observed in isolated

Abbreviations used: CPAE cells, calf pulmonary artery endothelial cells; CsA, cyclosporin A; DTT, dithiothreitol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MTP, mitochondrial permeability transition pore; TMRE, tetramethylrhodamine ethyl ester; $\Delta\Psi_m$, electrical potential gradient across mitochondrial inner membrane.

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organelles, demonstrating the feasibility of recording MTP openings and closings in intact cells. Prolonged depolarization was followed by swelling and breakdown of mitochondrial filaments.

MATERIALS AND METHODS

Isolation of mitochondria and experimental solutions

Heart mitochondria were isolated from rat ventricle by differential centrifugation [6] and suspended in a buffer solution containing (in mM): 225 mannitol, 75 sucrose, 20 Hepes, 0.5 EGTA; adjusted to pH 7.2 with KOH. Mitochondria were immobilized on a glass coverslip by centrifugation (5 min at 1800 ***g***). During the recordings the suspension buffer was replaced by KCl buffer containing (in mM): 140 KCl, 10 NaCl, 2 $MgCl_2$, 0.5 KH_2PO_4, 20 Hepes, 0.5 EGTA; adjusted to pH 7.2 with KOH. The KCl buffer was supplemented with 1 mg/ml rotenone and 10 mM succinate. TMRE (200 nM) (Molecular Probes) was added to all solutions. During the recordings, mitochondria were placed in the laminar flow from a solenoid-operated flow system allowing the rapid exchange of the extramitochondrial solution ($t_{\frac{1}{2}} \approx$ 100 ms). All experiments were performed at room temperature (20–22 °C).

Cultured vascular endothelial cells

Experiments were performed on single cultured calf pulmonary artery endothelial (CPAE) cells (CCL-209; American Type Culture Collection, Manassas, VA, U.S.A.) originally derived from bovine pulmonary artery endothelial cells. During experimentation, cells were superfused with Tyrode solution containing (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 10 Hepes; pH adjusted to 7.3 with NaOH. TMRE (200 nM) was added to the extracellular solution.

Fluorescence measurements

For fluorescence recordings the coverslip with the mitochondria or cultured CPAE cells was mounted on the stage of an inverted microscope (Axiovert 100; Carl Zeiss, Germany) equipped with a 40× oil-immersion objective (Plan-Neofluar, numerical aperture 1.3; Carl Zeiss). The microscope was attached to a confocal scanning unit (LSM 410; Carl Zeiss). TMRE fluorescence was excited at 514 nm with the attached argon-ion laser and was simultaneously recorded at 590 ± 15 nm. Time-dependent TMRE fluorescence changes were measured in the frame-scan mode of the microscope. Two-dimensional images (128 pixels × 128 pixels) were acquired at rates of 0.6–0.8 Hz with a total illumination time of 280 ms per image. Fluorescence was integrated over regions of interest (1 μm^2 or less), each placed over a single mitochondrion. Because the relationship between TMRE fluorescence and $\Delta\Psi_m$ is governed by the Nernst equation, fluorescence recordings are shown on a logarithmic scale.

RESULTS

Fast spontaneous fluctuations in $\Delta\Psi_m$ in single mitochondria underlie gradual voltage decay in mitochondrial populations

Figure 1 shows measurements of $\Delta\Psi_m$-dependent TMRE fluorescence derived simultaneously from three individual cardiac mitochondria. At the beginning of the recording the mitochondria were challenged with ADP (50 μM), causing a depolarization as a result of the electrogenic ATP/ADP exchange and the increased rate of H^+ backflow through the ATP synthase. Importantly, the

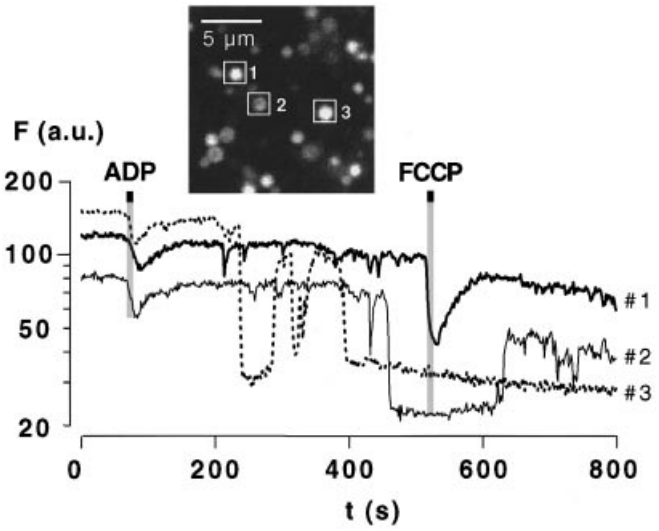

Figure 1 Spontaneous and experimentally evoked depolarizations in individual mitochondria

$\Delta\Psi_m$-dependent TMRE fluorescence (*F*) was recorded simultaneously from three individual mitochondria as indicated in the two-dimensional image. At the beginning of the recording mitochondria were stimulated by the rapid application of ADP (50 μM) resulting in a decrease in fluorescence, indicative of membrane depolarization. Removal of extramitochondrial ADP was followed by a complete recovery of $\Delta\Psi_m$. In contrast with the ADP-evoked response, which occurred simultaneously in all three organelles, after 200 s repetitive spontaneous depolarizations not synchronized between individual mitochondria were observed. Depolarizations were either of short duration and of smaller amplitude, or $\Delta\Psi_m$ dissipation lasted tens of seconds and reached levels not further sensitive to FCCP (0.5 μM). In this Figure and in Figures 4 and 5, the abbreviation a.u. means arbitrary units of fluorescence.

experimentally evoked decrease in $\Delta\Psi_m$ occurred simultaneously in all mitochondria. Mitochondria nos. 2 and 3 displayed rapid spontaneous depolarization to levels not sensitive to depolarization by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). In polarized mitochondria, the fast application of a maximally uncoupling concentration of FCCP (0.5 μM) caused a rapid and reversible depolarization (e.g. mitochondrion no. 1). Even after prolonged depolarizations (up to 150–200 s), the recovery of $\Delta\Psi_m$ was observed in some mitochondria (e.g. nos. 2 and 3). In addition to the large-amplitude prolonged voltage drops, the recordings revealed fast depolarizations of smaller amplitude and shorter duration. As demonstrated previously, these events were significantly less frequent in CsA-treated mitochondria and the time interval required for the irreversible dissipation of $\Delta\Psi_m$ increased approx. 3–4-fold after treatment with CsA [5]. It was therefore concluded that the fluctuations in $\Delta\Psi_m$ were due to short- and long-lasting openings of MTP.

Because of the stochastic nature of MTP openings in individual mitochondria, the comparison of $\Delta\Psi_m$ traces obtained from two single organelles under different conditions proved not to be useful to detect the stimulation or inhibition of MTP by drugs or other experimental variations. We therefore averaged recordings from individual mitochondria (20–50 organelles) under identical experimental conditions to obtain the 'ensemble response'. As shown in Figure 2(A), in the ensemble trace the rapid transition of the individual mitochondrion was no longer detectable and the voltage decay was gradual. The slope of the decay was used as a qualitative measure of the average rate with which mitochondria were recruited to undergo permeability transition. Similarly to measurements performed on mitochondrial popu-

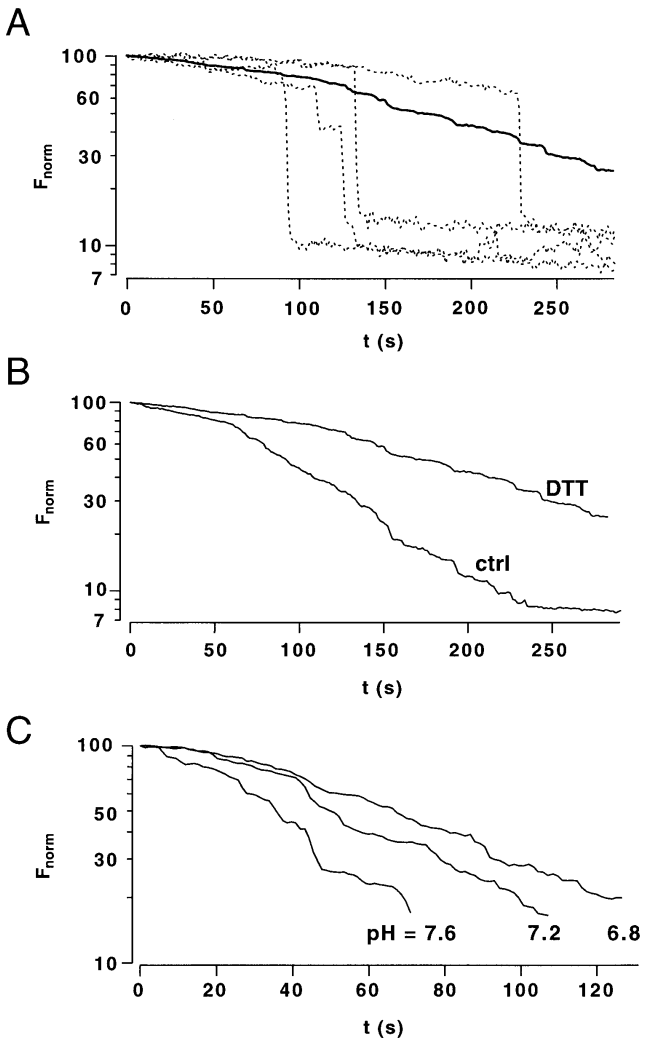

Figure 2 Effects of DTT and pH on voltage decay in mitochondrial ensembles

(**A**) To resolve the effects of drugs and other variations of experimental conditions on the rapid decreases in voltage in individual organelles, we calculated the 'ensemble response' (solid line) by averaging 20–40 traces obtained from single mitochondria under identical conditions. Before averaging, the fluorescence measured in single organelles at the beginning of the recording was normalized to 100 (F_{norm}). The dotted traces represent normalized fluorescence traces from four individual mitochondria. By analogy with population measurements, the slope of the voltage decay indicated the rate of recruitment of individual organelles to undergo permeability transition. (**B**) DTT slowed the voltage decay in mitochondrial ensembles. (**C**) Acidification protected mitochondria from light-induced voltage decay.

lations, the slope of the decay was readily affected by drugs well known to interfere with MTP [2] such as CsA, GSH and the free-radical scavengers butylated hydroxytoluene and catalase [5]. The following experiments provided additional support for the hypothesis that rapid fluctuations in $\Delta\Psi_m$ were indeed the result of openings and closings of MTP. Figure 2(B) shows an experiment comparing the voltage decay in a mitochondrial ensemble in the presence and the absence of the thiol-reducing agent DTT. As has been shown for the Ca^{2+}-induced MTP opening in mitochondrial suspensions [7–9], DTT provided marked protection against the illumination-induced voltage decay, indicating that the oxidation of thiols led to MTP opening. Figure 2(C) demonstrates the effect of pH (6.8–7.6) on the rate of

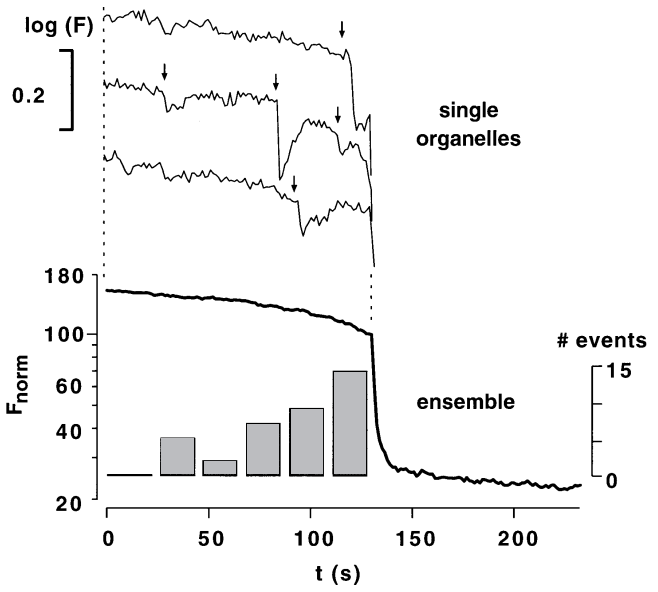

Figure 3 Two modes of MTP gating: short openings preceded prolonged openings

Twenty recordings from individual mitochondria obtained under control conditions were aligned in time and normalized in amplitude (F_{norm}) to the point at which the rapid large-amplitude voltage decrease occurred. The resulting ensemble trace is represented by the bold line. Before the voltage decrease, $\Delta\Psi_m$ declined gradually; the rate of depolarization increased with time. The bar diagram gives the temporal distribution of short-lasting small-amplitude depolarizations before the large voltage decrease (bin size 20 s). Example recordings of small-amplitude transient depolarization (arrows) in individual organelles (thin traces) are shown in the top panel. Abbreviation: #events, number of events.

$\Delta\Psi_m$ dissipation. Acidification has been shown to effectively counteract the permeability transition under all experimental conditions [1,2] and the protonation of crucial matrix-facing histidine residues has been identified as the underlying molecular mechanism [10]. In agreement with these reports, acidification of the bathing solution slowed the fluorescence decay significantly in the ensemble of isolated mitochondria (Figure 2C).

Short-lasting low-amplitude depolarizations precede large-amplitude voltage decreases

We have previously shown [5] that the large-amplitude voltage decreases are caused by openings of the transition pore. However, the nature of the short-lasting depolarizations of lower amplitude (see Figure 1) has not yet been clearly resolved. Because of their rapid kinetics and depressed frequency in the presence of CsA, we hypothesized that they might be the result of very brief openings of MTP. To examine further the possible connection between the fast low-amplitude and the prolonged large-amplitude depolarizations we analysed the temporal relationship between the two types of event; the result is shown in Figure 3. The bold trace shows an average recording obtained from 20 individual mitochondria under identical conditions. The traces from individual mitochondria have been aligned in time and normalized in amplitude at the time point when the rapid large-amplitude voltage decrease occurred (arrow). The bars in the graph below give the frequency of transient small-amplitude depolarizations before the large voltage decrease (bin size 20 s). From this experiment it was apparent that transient short-lasting

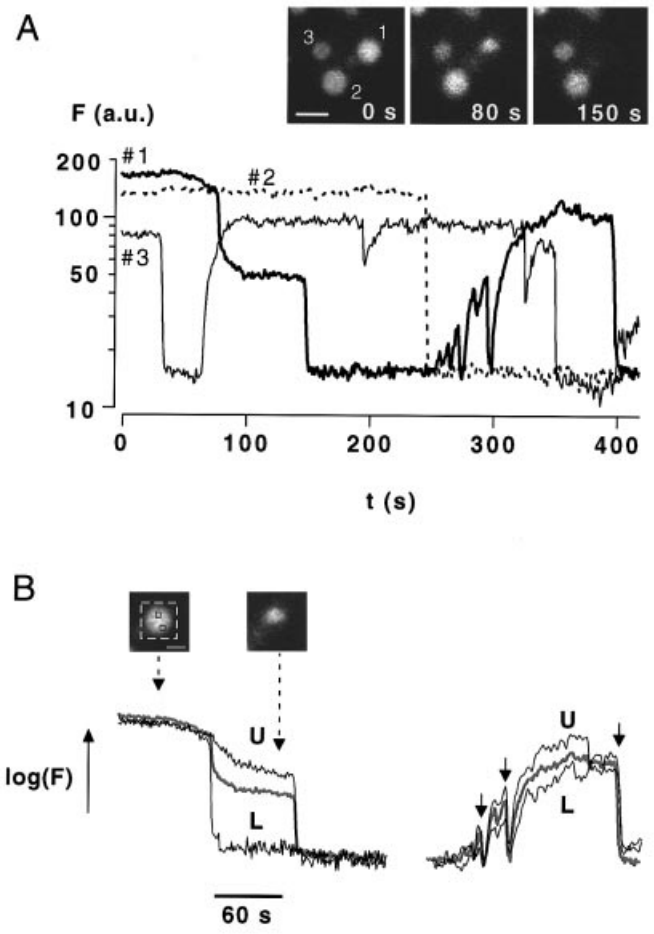

Figure 4 Two-step depolarization in subcompartmentalized mitochondria

(**A**) Simultaneous recording of TMRE fluorescence in three individual mitochondria. (**B**) Time course of $\Delta\Psi_m$-dependent TMRE fluorescence in the upper (U) and lower (L) hemispheres of mitochondrion no. 1 during depolarization and recharging. The bold trace represents the signal averaged over the entire organelle.

depolarizations increased in frequency before the long-lasting depolarization, i.e. prolonged pore opening (or sustained flickering). This time-dependent increase in the frequency of short events resulted in the slow but steadily increasing depolarization that preceded the large-amplitude transition of the ensemble trace.

Two-step depolarization revealed mitochondrial subcompartmentation

In 16 of approx. 300 mitochondria, the large-amplitude depolarization occurred in two discrete steps. $\Delta\Psi_m$ initially decreased to a lower level and remained stable before the mitochondrion eventually discharged completely in a second rapid step. Figure 4(A) shows fluorescence traces obtained simultaneously from three single mitochondria. Fluorescence was averaged over a region of interest including the entire mitochondrion. $\Delta\Psi_m$ in mitochondrion no. 2 (broken line) was stable for a period of approx. 250 s, after which the dye was rapidly lost, indicating MTP opening and sudden depolarization. Mitochondrion no. 3 discharged rapidly after 40 s. After approx. 30 s, the low membrane permeability was restored and $\Delta\Psi_m$ recovered completely. After repolarization, two short-lasting voltage transients were observed before $\Delta\Psi_m$ eventually dissipated again. Thus mitochondria nos. 2 and 3 displayed the typical behaviour of TMRE-stained organelles during prolonged fluorescence recordings. Mitochondrion no. 1, however, discharged in two discrete steps. The voltage changes in mitochondrion no. 1 were analysed in more detail in Figure 4(B). After the initial partial depolarization, fluorescence was restricted to the upper hemisphere of the organelle, indicating a functional subcompartmentation of mitochondrion no. 1. This interpretation was supported by a comparison of the time course of changes in $\Delta\Psi_m$ in the upper (U) and lower (L) hemispheres. The rapid depolarization was initially limited to the lower compartment while $\Delta\Psi_m$ in the upper half of the organelle decreased only slowly. After approx. 60 s, however, the upper compartment also discharged rapidly. At 100 s after the second decrease in voltage, $\Delta\Psi_m$ in both hemispheres recovered almost to its initial level. The recovery was interrupted by brief depolarizations. Interestingly, during the recovery period no indications of two stable potential levels were observed. Moreover, $\Delta\Psi_m$ in the previously isolated compartments had the same time course, indicating that they were electrically coupled during the recharging process. Therefore the rapid voltage decrease in the upper hemisphere (Figure 4B) might reflect membrane fusion and resulting electrical continuity of the two subcompartments rather than an opening of MTP in the second, upper, compartment. The possibility had to be considered that mitochondrion no. 1 was not a single organelle but two separate mitochondria. The ideal spherical shape of mitochondria nos. 1–3 after isolation, however, indicated that a single outer membrane surrounded each of the three mitochondria. Thus in some organelles a single outer membrane enveloped two electrically isolated matrix compartments. It remains unknown whether these organelles are mitochondria in the process of division or fusion [11,12] or whether the subcompartmentation resulted from the 'artificial' formation of intramitochondrial septa [13]. Importantly, the coupling between subcompartments was dynamic, switching between coupled and isolated on a fast (tens of seconds or faster) time scale.

$\Delta\Psi_m$ fluctuations in individual mitochondria in the living cell

The recordings presented so far demonstrate that in individual isolated mitochondria $\Delta\Psi_m$ displayed spontaneous rapid fluctuations that were caused by stochastic openings of MTP. To verify that the fluctuations in the transmembrane voltage gradient were not caused by some artifact of isolation, we attempted to measure changes in $\Delta\Psi_m$ in individual organelles in intact cells. However, measurements of $\Delta\Psi_m$ in single mitochondria within the cell are in many cases significantly interfered with by organelle motility [14]. Organelle transport along microtubules or changes in shape [12] can result in fluorescence changes that are independent of changes of $\Delta\Psi_m$. We found this problem to be less severe when imaging single organelles in extremely flat regions at the very edge of cultured endothelial cells. However, more than 75% of the recordings revealed clear signs of mitochondrial motion and were discarded from analysis. Figure 5(A) shows a representative recording of the voltage-sensitive TMRE fluorescence obtained simultaneously from two mitochondria in a cultured vascular endothelial cell. No motion was detected in these organelles over the period of recording (260 s). The fluorescence trace revealed marked spontaneous fluctuations in $\Delta\Psi_m$ between the charged and discharged state that were virtually identical with the voltage fluctuation observed in isolated mitochondria. Importantly, the voltage fluctuations were not

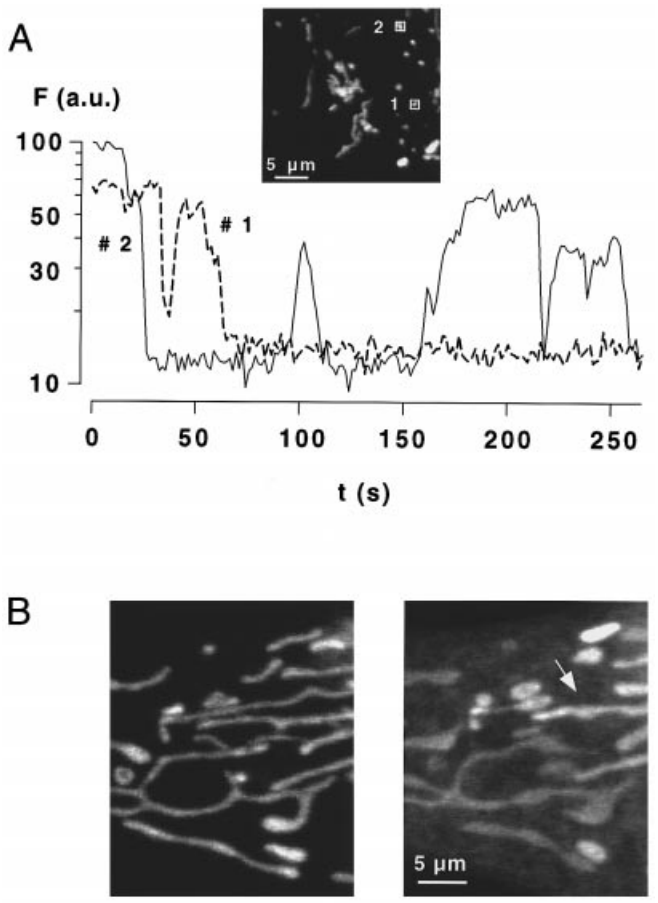

Figure 5 Spontaneous fluctuations in membrane potential in individual mitochondria *in situ*

(**A**) Time course of $\Delta\Psi_m$ in two individual mitochondria in a cultured vascular endothelial cell. (**B**) TMRE-stained endothelial cell mitochondria at the beginning (left panel) and after a period of intense laser illumination (right panel). The offset and gain of the image in the right panel have been adjusted to optimize the visibility of organelles (note the increased cytosolic background fluorescence). Fluorescence intensity levels between the two images therefore cannot be directly compared with each other. After exposure to intense laser illumination, mitochondria appeared swollen. In some cases (arrow), fragmentation of mitochondrial filaments was observed.

synchronized between individual mitochondria in the cell. This observation ruled out changes in cell-surface membrane potential from underlying the changes in mitochondrial fluorescence. Any change in the cell membrane potential would result in changes of the cytosolic dye concentration and would therefore affect the mitochondrial concentration independently of changes in $\Delta\Psi_m$. However, such changes are expected to affect the entire cellular population of mitochondria equally. Further control experiments (results not shown) showed that $\Delta\Psi_m$ in cellular populations of mitochondria gradually depolarized with time. Similarly to the ensemble behaviour of isolated cardiac mitochondria, the decay of TMRE fluorescence was accelerated by increasing light exposure per unit time or by increasing the dye concentration (results not shown).

In Figure 5(B), two confocal images of TMRE-stained mitochondria in a cultured CPAE cell before (left panel) and after (right panel) an extended period of laser illumination are shown. The comparison of the two images revealed marked structural changes in the mitochondrial morphology after light-induced oxidative stress. The organelles were typically filamentous, sometimes with multiple branching points. Some smaller, more spherical, mitochondria were also observed. After a period of constant laser illumination of the cell (2 min), mitochondrial filaments shortened and appeared swollen. In some instances breakdown of filamentous mitochondria was observed (Figure 5B, right panel, arrow). Fragmentation of mitochondrial filaments was fast, taking only a few seconds. Therefore prolonged exposure of mitochondria in the cellular environment to laser illumination resulted in functional damage (dissipation of $\Delta\Psi_m$) and structural damage (swelling and breakdown of mitochondrial filaments) of the organelles. Because of the comparable kinetics of depolarizations observed in single mitochondria after isolation and in the intact cell, the same underlying trigger (photo-induced oxidative stress), and the permeability increase indicated by swelling of the organelle, we suggest that the voltage fluctuations in both single mitochondria *in situ* and in isolated organelles were caused by repetitive openings of MTP.

DISCUSSION

Twenty years ago, Haworth and Hunter [1] introduced the pore concept for the Ca^{2+}-induced permeabilization process of the inner mitochondrial membrane. Their hypothesis was based on the observation that (1) the permeability pathway had a very sharp size cut-off for permeant solutes (1500 Da or less) and (2) the ultrastructural transition from the 'aggregated' (or contracted) to 'expanded' (or orthodox) configuration caused by the increase in permeability seemed to be 'all or none' at the level of single organelles. The pore model was further supported by the identification of a Ca^{2+}-activated and CsA-sensitive large-conductance ion channel in patch clamp experiments performed on inner mitochondrial membranes [2]. We recently reported spontaneous rapid fluctuations in transmembrane voltage in single isolated mitochondria that coincided with increases in the permeability for matrix-entrapped calcein (620 Da) and were decreased by CsA [5]. The rapid kinetics of $\Delta\Psi_m$ dissipation in the single organelle together with the repetitive recharging of the inner membrane were best explained by repetitive openings and closings of a membrane pore, i.e. the MTP. In this study we provide further evidence that the rapid depolarizations in single mitochondria coincided with openings of MTP (inhibition by DTT and acidification; Figure 2) and we carefully analysed the permeabilization/depolarization process in single organelles. Moreover, we demonstrated that similar voltage fluctuations occur in individual mitochondria in the living cell.

Mitochondrial subcompartmentation

In the vast majority of mitochondria, MTP opening resulted in the rapid loss of the potentiometric fluorescent dye from the matrix compartment. Dye efflux followed a time course described by a single exponential with a half time of 1 s or less. This value is likely to be limited by the kinetics of dye redistribution in response to changes in $\Delta\Psi_m$. In rare instances (approx. 5% of organelles) the decrease in fluorescence occurred in two discrete steps. High-resolution imaging of TMRE-stained mitochondria (Figure 4) revealed a functional subcompartmentation underlying the two-step depolarization. Tandler and Hoppel [11] have identified partitioned mitochondria containing two separate matrix compartments in cardiac muscle from several species. Moreover, mitochondrial partitioning was conserved after isolation. The authors interpreted the intramitochondrial partitions as being the result of division. However, mitochondrial internal septa have also been shown to be experimentally inducible by

subjecting cells and mitochondria to massive Ca^{2+} loads [13]. The possibility that mitochondria might rapidly form internal septa in response to stress, e.g. high Ca^{2+} loads or radical formation, might provide a mechanism for minimizing damage caused by MTP. Moreover, a functional subcompartmentation of mitochondria in neuroblastoma cells has been suggested on the basis of the observation of pH gradients within single organelles [15]. However, a possible functional significance of such mitochondrial subcompartments has yet to be established. Nevertheless, the small size of the average cardiac mitochondrion and the remarkable turnover rate of this organelle (average life time approx. 4–5 days) [16] would suggest that the mitochondrial partitioning most probably resulted from divison.

Properties of MTP in single organelles

The experiments performed on isolated heart mitochondria further revealed that short-lasting depolarizations of smaller amplitude frequently preceded the large-amplitude voltage decrease (Figure 3). On the basis of their rapid kinetics and sensitivity to CsA, we have suggested that these events are caused by brief openings of MTP. Calcein (620 Da) efflux associated with sustained mitochondrial depolarization after rapid dissipation of $\Delta\Psi_m$ was relatively slow ($t_{\frac{1}{2}} \approx 10$ s) [5]. Therefore short-lasting depolarizations were not paralleled by any measurable efflux of matrix-entrapped calcein [5]. Similar rapid transient depolarizations were observed in mitochondria in intact vascular endothelial cells (Figure 5). The probability of brief MTP openings increased with the duration of oxidative stress before prolonged opening(s) dissipated $\Delta\Psi_m$ without recovery (Figure 4). Generalizing this notion, we suggest a 'gradual permeabilization process' in the single mitochondrion in response to MTP-inducing stimuli (Ca^{2+} or oxidative stress). During periods of stress, MTP initially opens very briefly, allowing a flux of ions (H^+, K^+, Mg^{2+} and Ca^{2+}), whereas larger solutes, e.g. adenine or pyridine nucleotides, are largely retained. As a result, $\Delta\Psi_m$ and matrix [Ca^{2+}] are decreased, thereby counteracting the potentially damaging consequences of prolonged matrix Ca^{2+} overload and/or very high $\Delta\Psi_m$ [17]. Furthermore, brief openings of MTP limit the duration of uncoupling of oxidative phosphorylation from respiration and thereby prevent cellular ATP depletion. However, prolonged stress eventually causes MTP to open for longer periods, resulting in the complete equilibration of electrical and concentration gradients. The pronounced fluctuations in $\Delta\Psi_m$ seen in individual mitochondria *in vitro* (Figure 1) and *in vivo* (Figure 5) demonstrated spontaneous MTP closures and recharging of the inner membrane even after longer periods of pore opening (up to 150 s) and maintained the oxidative stress imposed experimentally by laser irradiation. Most mitochondria (85 %), however, displayed no recovery after the rapid and complete discharge of $\Delta\Psi_m$. Thus brief openings of MTP might serve as an emergency mechanism to rid mitochondria of excess Ca^{2+} or to decrease the rate of endogenous radical formation by lowering $\Delta\Psi_m$ [17]. In contrast, a loss of matrix components and structural damage (e.g. swelling; see Figure 5) render prolonged pore openings deleterious to mitochondria. When measured in mitochondrial populations respiring in sugar media, the experimentally undetectable flux of large molecules (such as sucrose or calcein) during brief MTP openings would result in the dissipation of $\Delta\Psi_m$, the release of Ca^{2+}, Mg^{2+} and K^+ without significant entry of sugar, and subsequent swelling. Reports that K^+ and Mg^{2+} efflux [18], H^+ permeation [19] and membrane depolarization [20] precede the entry of sugar and mitochondrial swelling under certain experimental conditions have been interpreted as evidence for possible solute-selective sublevels of MTP. These results are also compatible with the notion of a 'modal MTP gating', i.e. MTP openings during mitochondrial stress are initially very brief, resulting in the equilibration of ionic gradients before prolonged openings cause sugar entry and massive swelling of the matrix compartment.

MTP in the living cell

Rapid fluctuations in $\Delta\Psi_m$ with very similar kinetics compared with those in isolated mitochondria were also observed in individual organelles in living cells (Figure 5). The same mechanism of induction (i.e. the light-induced generation of free radicals) and the same fast kinetics of the depolarizations strongly suggested that MTP underlies the mitochondrial voltage decay *in situ*. Interestingly, however, CsA (0.2–2 μM) had no effect on the mitochondrial depolarizations in intact endothelial cells. Although inhibition by CsA is often used to identify MTP, there are several reports documenting a limited effect, or even a lack of effect, on MTP by CsA [2]. Furthermore, the abundance of CsA-binding proteins in different subcompartments of intact cells makes this observation less surprising and potentially limits the usefulness of this drug as a 'specific' MTP antagonist in the cellular environment.

Similarly to our study, Amchenkova et al. [21] used laser point illumination of rhodamine-stained organelles to induce local 'mitochondrial damage'. Although the mechanism underlying the light-induced depolarization was not known then, it is noteworthy that these authors reported the occasional recovery of $\Delta\Psi_m$ in laser-damaged mitochondria. The time-resolved measurements of $\Delta\Psi_m$ in individual mitochondria inside the cell reported in the present paper revealed repetitive depolarizations. Thus transient opening(s) and closing(s) of MTP also seemed to be a property of the transition pore inside the cellular environment. As a result of prolonged illumination and sustained MTP opening, mitochondria appeared swollen. In some cases we observed fragmentation of mitochondrial filaments (Figure 5B). A similar swelling and fragmentation of filamentous mitochondria in human osteosarcoma cells in response to MTP opening has been reported by Minamikawa et al. [22].

Experimental evidence is accumulating for the involvement of MTP in various forms of cell injury, most prominently during ischaemia–reperfusion in heart and liver [23]. By using the entrapment of [^{3}H]deoxyglucose in the mitochondrial compartment to follow MTP openings *in situ*, Griffiths and Halestrap [24] demonstrated that in perfused rat hearts the non-specific mitochondrial pores remained closed during global ischaemia but readily opened after reperfusion (see also [25]). This observation further strengthens the role of oxidative damage in the mitochondrial permeability transition. It also shows that experimental systems in which MTP is induced through oxidative stress might provide useful information about the underlying pathophysiological mechanisms. MTP also constitutes an important step in some tightly regulated pathways resulting in programmed cell death [26]. However, it is controversial whether transient openings of MTP occur under physiological conditions. Ichas et al. [27] have suggested that MTP operating in a low-conductance mode regulated by matrix pH participates in cellular Ca^{2+} signalling. Convincing evidence for such a mechanism in intact cells is lacking. Furthermore, Petronilli et al. [28] demonstrated a slow CsA-sensitive release of calcein preloaded into the mitochondrial matrix and interpreted these results as evidence for transient MTP openings in intact cells under resting conditions. In our view, however, MTP seems to be a phenomenon restricted to extreme conditions of cellular Ca^{2+} overload and/or

oxidative stress that is most probably encountered only under pathophysiological conditions.

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