Imaging the Permeability Pore Transition in Single Mitochondria

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ABSTRACT In mitochondria the opening of a large proteinaceous pore, the "mitochondrial permeability transition pore" (MTP), is known to occur under conditions of oxidative stress and matrix calcium overload. MTP opening and the resulting cellular energy deprivation have been implicated in processes such as hypoxic cell damage, apoptosis, and neuronal excitotoxicity. Membrane potential ($\Delta\Psi_m$) in single isolated heart mitochondria was measured by confocal microscopy with a voltage-sensitive fluorescent dye. Measurements in mitochondrial populations revealed a gradual loss of $\Delta\Psi_m$ due to the light-induced generation of free radicals. In contrast, the depolarization in individual mitochondria was fast, sometimes causing marked oscillations of $\Delta\Psi_m$. Rapid depolarizations were accompanied by an increased permeability of the inner mitochondrial membrane to matrix-entrapped calcein (≈620 Da), indicating the opening of a large membrane pore. The MTP inhibitor cyclosporin A significantly stabilized $\Delta\Psi_m$ in single mitochondria, thereby slowing the voltage decay in averaged recordings. We conclude that the spontaneous depolarizations were caused by repeated stochastic openings and closings of the transition pore. The data demonstrate a much more dynamic regulation of membrane permeability at the level of a single organelle than predicted from ensemble behavior of mitochondrial populations.

INTRODUCTION

The electrical potential difference ($\Delta\Psi_m$) across the inner mitochondrial membrane together with the H^+ concentration gradient (ΔpH) constitute the driving force for ADP phosphorylation catalyzed by ATP synthase (F_0F_1-ATPase). Both $\Delta\Psi_m$ and ΔpH are ultimately established and maintained by the redox-driven H^+ pumps of the respiratory chain (Mitchell, 1966; Nicholls, 1982). To keep up the high rates of ATP synthesis in the living cell, the resting permeability of the energy-transducing (inner) membrane is extremely low. Under certain conditions, however, the opening of a large proteinaceous pore, referred to as mitochondrial permeability transition pore (MTP), renders the inner membrane permeable to molecules up to 1500 Da (Haworth and Hunter, 1979; Gunter and Pfeiffer, 1990; Gunter et al., 1994; Bernardi et al., 1994; Zoratti and Szabo, 1995). Opening of the MTP results in the rapid collapse of the membrane potential, equilibration of ion gradients, and loss of metabolites (e.g., nucleotides). Many of its characteristics point to MTP and the subsequent energy (ATP) deprivation of the cell as fundamental mechanisms for cell injury and cell death following ischemia/reperfusion (Halestrap, 1994) or as a consequence of glutamate excitotoxicity (White and Reynolds, 1996). Moreover, it has been demonstrated that MTP constitutes a fundamental step in the signaling cascade leading to programmed cell death (Zanzami et al., 1996). MTP opening is triggered by Ca^{2+} overload of the matrix compartment in conjunction with various agents referred to as "inducing agents" (Gunter and Pfeiffer, 1990). In addition, the immunosuppressant drug cyclosporin A has been identified as a potent inhibitor of the pore transition (Crompton et al., 1988; Broekemeier et al., 1989). By using the patch clamp technique a multiconductance ion channel (MCC) has been found in mitoplasts, i.e., the inner mitochondrial membrane. Its large conductance (≥1 nS), Ca^{2+}-dependence, and sensitivity to cyclosporin A renders the channel a likely candidate for MTP (Szabo and Zoratti, 1992). Current models for the transition pore propose that the pores reside in "contact sites" between outer and inner mitochondrial membrane. This notion is supported by the finding that MCC activity has been recorded following reconstitution of contact sites into liposomes or planar lipid bilayer (Moran et al., 1990; Beutner et al., 1996). Because of the close association of kinases, the mitochondrial porin (or voltage-dependent anion channel; VDAC), and the ATP/ADP carrier of the inner membrane within contact sites these structures have been implicated in the regulation of oxidative phosphorylation (see Brdiczka, 1991 for a review). Furthermore, a large body of evidence suggests an important role of contact sites in the process of mitochondrial protein import (Brdiczka, 1991; Pfanner et al., 1990). To date, there is no agreement on what protein(s) form the transition pore. Szabo and co-workers (Szabo and Zoratti, 1993; Szabo et al., 1993) suggested that the pore might result from the cooperative gating of two VDAC channels located in the inner membrane. Other models (e.g., Halestrap, 1994) suggest the involvement of the ATP/ADP carrier. The latter are supported by the observation that the reconstituted carrier forms large ion channels when treated with thiol reagents (Tikhonova et al., 1994) or high Ca^{2+} (Brustovetsky and Klingenberg, 1995).

MTP has been most extensively studied in suspensions of isolated mitochondria. Because of the signal averaging over

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a large population of mitochondria in these experiments, MTP is evident as a gradual process, e.g., loss of membrane potential or release of Ca^{2+}. The transition of an individual mitochondrion, however, is believed to be very fast and the gradual response recorded from mitochondrial populations is caused by the successive recruitment of organelles undergoing pore transition (Gunter et al., 1994). Thus, important information on the kinetics of the transition process cannot be resolved in population measurements. In contrast, electrophysiological studies on MCC activity reveal many useful kinetic characteristics of the transition pore. These experiments need, however, to disrupt the complex membrane structure of the mitochondrion. The preparation of mitoplasts or reconstitution of contact sites might result in the loss of protein or nonprotein components involved in the regulation of MTP. Therefore, the experimental results do not necessarily reflect the behavior of MTP in the intact organelle.

In the experiments presented here we have used laser scanning confocal microscopy in combination with the potentiometric fluorescence dye tetramethylrhodamine ethyl ester (TMRE) (Farkas et al., 1989; Loew et al., 1993) to monitor relative changes in membrane potential in single isolated cardiac mitochondria that have been immobilized on a glass coverslip. This cationic dye distributes across the membrane in a voltage-dependent manner governed by the Nernst equation. Therefore, the large potential gradient across the inner mitochondrial membrane results in the accumulation of the fluorescent dye within the matrix compartment. The experimental setup allowed rapid exchange of the extramitochondrial solution ($t_{0.5} \approx 100$ ms) while fluorescence was recorded simultaneously from up to 10 individual mitochondria. The recordings revealed marked fluctuations in membrane potential that were paralleled by an increased permeability of the inner membrane to large molecules consistent with pore transition.

MATERIALS AND METHODS

Isolation of mitochondria and solutions

Mitochondria were isolated from rat ventricle by differential centrifugation (Palmer et al., 1977). The final suspension buffer contained (in mM): 225 mannitol, 75 sucrose, 20 HEPES, 0.5 EGTA, pH = 7.2 (KOH). Before experimentation mitochondria were immobilized on a glass coverslip by centrifugation (5 min at 1800 × *g*). For experimentation the suspension buffer was replaced by KCl buffer containing (in mM): 140 KCl, 10 NaCl, 2 $MgCl_2$, 20 HEPES, 0.5 KH_2PO_4, 0.5 EGTA, pH = 7.2 (KOH). The buffer was supplemented with 1 mg/l rotenone and 10 mM succinate. All solutions contained 0.2 μM TMRE. Experimental results were similar when the high ionic strength buffer was replaced by a mannitol/sucrose based solution. The $\Delta\Psi_m$ decay of the mitochondrial population, however, was found to be slower in sugar medium without changing the rapid kinetics of the transition in single mitochondria (not shown). During the recording mitochondria were placed in the laminar flow of a solenoid-operated flow system, which allowed rapid solution exchanges ($t_{0.5} \approx 100$ ms).

Fluorescence measurements

For fluorescence measurements the coverslip with the mitochondria was mounted on the stage of an inverted microscope equipped with a 40× oil immersion objective (Plan-Neofluar, NA = 1.3, Carl Zeiss). The microscope was attached to a confocal laser scanning unit (LSM 410, Carl Zeiss, Germany). TMRE fluorescence was excited at 514 nm using the attached 25 mW argon-ion laser while simultaneously recorded at 590 nm. For measurements of the time-dependent TMRE fluorescence changes images were acquired every 1.0–1.5 s. The total illumination time was 280 ms per image. Fluorescence was integrated over up to 10 regions of interest (ROIs) $\leq 1\ \mu m^2$ (12 × 12 pixel), each placed over a single mitochondrion. Because the fluorescence intensity (F) was averaged during the recording over ROIs including different fractions of nonmitochondrial volume, different absolute values of F or ΔF among different mitochondria do not necessarily translate to differences in $\Delta\Psi_m$. Generally, the relationship between F and $\Delta\Psi_m$ is described by the Nernst equation. Therefore, to linearize the fluorescence changes for changes in $\Delta\Psi_m$, F is shown on a logarithmic scale. For the co-staining experiments calcein was introduced into the matrix compartment by exposing mitochondria to the membrane-permeant ester form (calcein/AM; 5 μM in KCl buffer). Fluorescence was excited at 488 nm and simultaneously measured at 515–525 nm (calcein) and 590 nm (TMRE).

RESULTS

Spontaneous rapid depolarizations in single mitochondria

Fig. 1 shows a typical recording of TMRE fluorescence obtained simultaneously from two single mitochondria. In all experiments mitochondria were energized using succinate as the respiratory substrate. During the recording illustrated in Fig. 1 the mitochondria were stimulated with 50 μM ADP. Upon addition of ADP a fraction of the transmembrane electrical potential was consumed for ATP synthesis by the mitochondrial ATP synthase. In contrast to measurements on mitochondrial suspensions in a closed experimental chamber, extramitochondrial [ADP] was kept constant by the perfusion system. Following washout of ADP, $\Delta\Psi_m$ recovered to its initial value. Importantly, the response of neighboring mitochondria to superfusion with ADP (Figs. 1 and 2), carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone (FCCP), or Ca^{2+} (not shown) was always simultaneous. However, spontaneous unsynchronized rapid and transient drops of $\Delta\Psi_m$ (*arrows* in Fig. 1) could be observed in individual mitochondria. In addition to these transient depolarizations we have regularly measured depolarizations with the same fast rate but larger amplitude and of much longer duration (Fig. 2). In these mitochondria $\Delta\Psi_m$ dropped to levels essentially insensitive to further depolarization by ADP (or FCCP, not shown) indicating complete uncoupling of respiration from ADP phosphorylation. Furthermore, Fig. 2 shows that before the mitochondria eventually remained depolarized, $\Delta\Psi_m$ fluctuated between the energized (ADP-sensitive) and discharged (ADP-insensitive) state. Removal of the metabolic substrate succinate following the initial drop of $\Delta\Psi_m$ prevented the recharging of the mitochondria indicating that the recovery depended on the activity of the proton pumps of the respiratory chain. The repetitive recovery of $\Delta\Psi_m$ suggests that

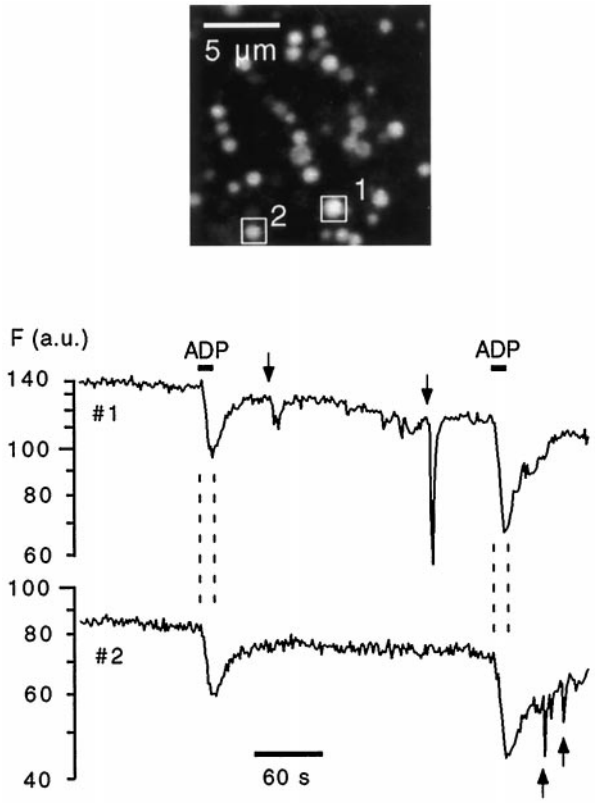

FIGURE 1 $\Delta\Psi_m$-dependent TMRE fluorescence changes recorded from single mitochondria. In two-dimensional confocal images (*top*) isolated immobilized mitochondria appeared as spherical fluorescent particles with an average diameter of 1.1 μm. During the recording mitochondria were challenged with 50 μM ADP resulting in a decrease of TMRE fluorescence (F) indicating mitochondrial membrane depolarization. The depolarization occurred simultaneously in all mitochondria monitored. The arrows mark fast transient depolarizations not synchronized in neighboring mitochondria.

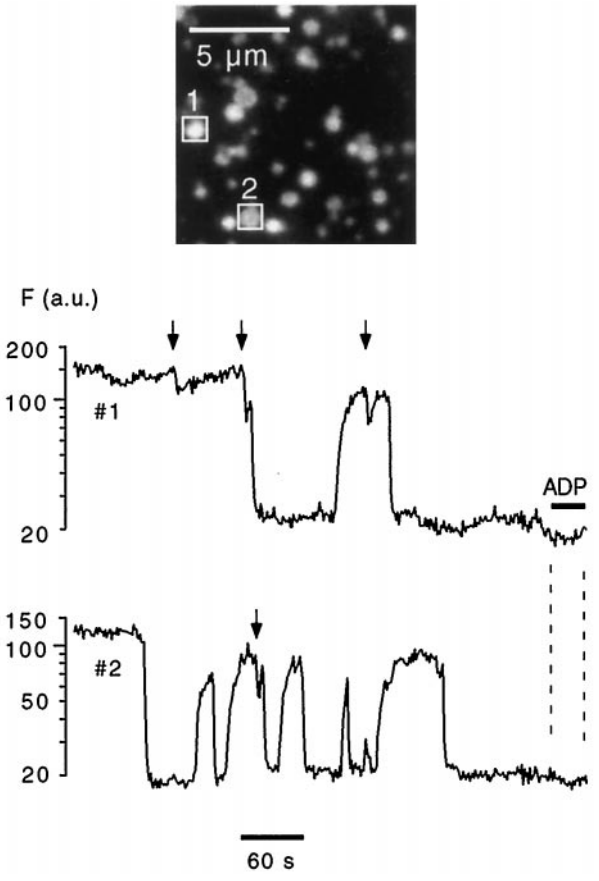

FIGURE 2 Rapid loss and large amplitude fluctuations of $\Delta\Psi_m$. In addition to the fast transient depolarizations (*arrows*) mitochondria frequently displayed voltage drops to levels where application of ADP (or FCCP, not shown) caused no further decrease of TMRE fluorescence. The depolarization was fast with $t_{0.5} \approx 2$ s. Mitochondria remained depolarized for variable periods. In many organelles, after the initial voltage drop, the low membrane permeability was restored, resulting in a recharging of the energy-transducing membrane. Recharging depended on the presence of an energy substrate (succinate in our experiments) and had a hyperbolic time course. Thus, fluctuations of membrane permeability gave rise to pronounced $\Delta\Psi_m$ oscillations before the mitochondria eventually remained depolarized.

the permeability of the inner mitochondrial membrane fluctuated between a low and high permeability state. Marked oscillations of $\Delta\Psi_m$ were observed in ~10–15% of the mitochondria. In these cases, after a few cycles of discharging and recharging, $\Delta\Psi_m$ remained eventually depolarized. In most mitochondria, however, the large amplitude voltage drop was not followed by any discernible recovery.

Effect of light-generated reactive oxygen species

The membrane permeability increase underlying the voltage drop in a single mitochondrion appeared to be a stochastic process, i.e., the time from the onset of the recording until the voltage drop occurred varied from mitochondrion to mitochondrion under otherwise identical conditions. This variability most likely reflected qualitative differences between individual organelles, such as the absolute membrane potential or level of endogenous NAD(P)H. It was therefore not possible to compare the effects of different drugs or recording conditions directly between individual mitochondria. In analogy to measurements on mitochondrial suspensions, we averaged the responses measured in individual organelles ($20 \leq n \leq 40$) under identical conditions to obtain the "ensemble response" (Fig. 3 *A*). The resulting trace revealed a striking similarity to population measurements in that the permeability transition was no longer evident as a sudden potential drop but as a gradual decline of the voltage-dependent fluorescence. In the example shown, the "transition" phase was preceded by a "lag" phase of ~1 min during which $\Delta\Psi_m$ remained fairly constant. With the potential drop in the first mitochondrion of the ensemble and the subsequent succession of further organelles the averaged fluorescence gradually declined. The rate of this successive recruitment of individual mitochondria was reflected in the slope of the decline of the "ensemble trace."

Illumination of a small region of the coverslip resulted in the progressive decline of $\Delta\Psi_m$ in all mitochondria exposed to the laser light with the transition being fast in the individual organelle. Mitochondria in neighboring unexposed

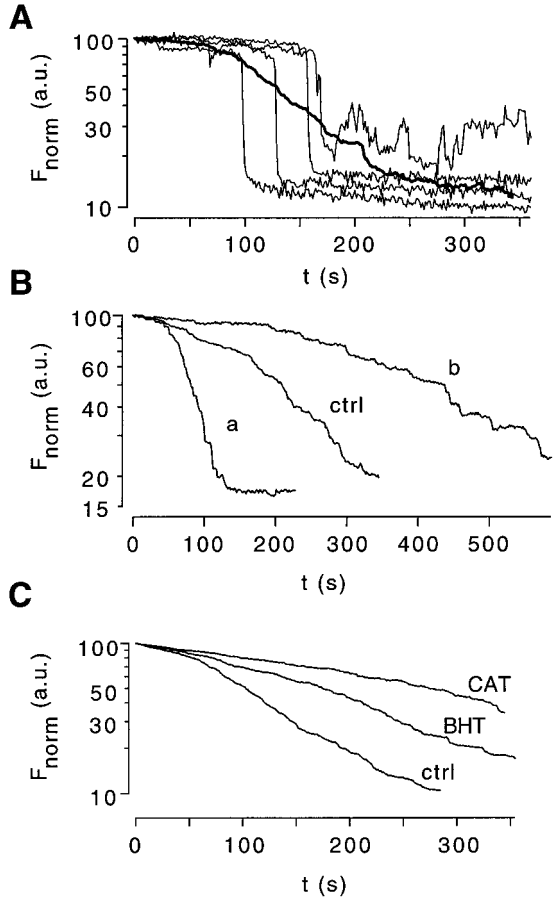

FIGURE 3 Effect of illumination and dye concentration on "ensemble behavior." (*A*) Comparison of recordings from individual organelles (*thin lines*) and the average ("ensemble") trace calculated from 20 mitochondria (*bold line*). (*B*) Ensemble traces under control condition (ctrl, $n = 40$), in the presence of higher dye concentration (*trace a:* 0.8 μM TMRE instead of 0.2 μM under control, $n = 32$), and following reduction of the illumination time (*trace b*, $n = 32$). (*C*) Slowing of the voltage decay in the mitochondrial ensemble ($n = 40$ for all traces) by the free radical scavenger BHT (20 μM) or catalase (CAT, 0.5 mg/ml).

regions, however, were totally unaffected (not shown), indicating that the damage depended on laser illumination. Fig. 3 *B* documents that the voltage decline critically depended on the concentration of the fluorescence dye and the amount of excitation light experienced by the single mitochondrion. Raising either the TMRE concentration or the illumination time resulted in an acceleration of the mitochondrial permeabilization, and vice versa. Excitation of fluorescence molecules using high energy laser light is known to produce reactive oxygen species (ROS) resulting from the interaction of the long-lived triplet state of the excited dye molecule with molecular oxygen (Tsien and Waggoner, 1995; Foote, 1968). To test directly whether ROS formation was involved in the permeabilization process we examined the effect of the free radical scavenger butylhydroxytoluene (BHT). As illustrated in Fig. 3 *C*, the voltage decay in the ensemble response was significantly slowed in the presence of BHT. This protective effect of BHT could be overcome by increasing the dye concentration (not shown). A recent report (Gudz et al., 1997) has shown that BHT might prevent the mitochondrial pore transition by directly binding to the pore or a regulatory component rather than by radical scavenging. Thus, a more straightforward indication for the involvement of ROS, specifically H_2O_2, comes from the observation that addition of catalase to the incubation medium slowed the $\Delta\Psi_m$ decline even more dramatically (Fig. 3 *C*). These findings are in agreement with recent studies showing that the mitochondrial permeabilization can be prevented or delayed by radical scavengers and catalase (Carbonera and Azzone, 1988; Kowaltowski et al., 1996).

Rapid depolarizations were caused by openings of the transition pore

In Fig. 4 *A* the effects of cyclosporin A (CsA, 1 μM) and reduced glutathion (GSH, 20 mM) on the ensemble behavior are shown. The MTP inhibitor CsA markedly reduced the rate of the mitochondrial potential decay. Analysis of the recordings from single organelles revealed that this was due to a significant prolongation of the average time period (t_D)

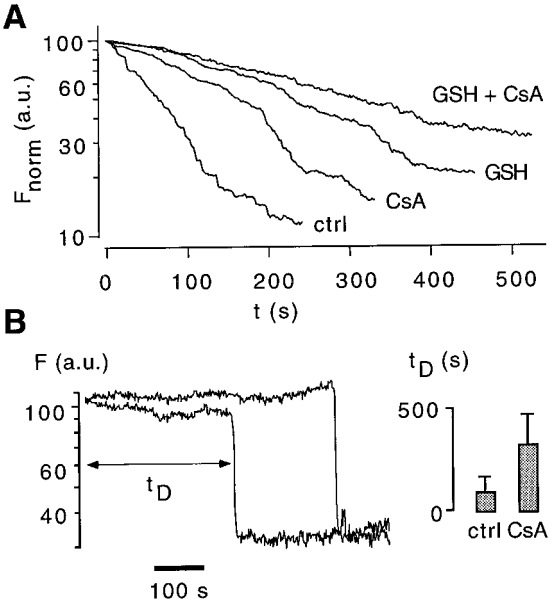

FIGURE 4 Effects of cyclosporin A and reduced glutathion on $\Delta\Psi_m$ decay. (*A*) Time course of normalized TMRE fluorescence in the presence or absence of 1 μM CsA and/or 20 mM GSH (substituted for an equimolar amount of KCl). The traces were derived by averaging recordings obtained from individual mitochondria ($n = 38$ for ctrl; $n = 33$ for CsA; $n = 35$ for GSH; $n = 33$ for GSH + CsA). To exclude qualitative differences between mitochondrial preparations, the measurements were performed on mitochondria of the same preparation within 2 h after isolation. (*B*) TMRE fluorescence recorded simultaneously in two different mitochondria in the presence of CsA. The right-hand panel compares the averaged time from the beginning of the recording to the $\Delta\Psi_m$ drop (t_D). The average t_D was 92 ± 72 s under control conditions and 322 ± 149 s in the presence of CsA (mean $\pm$ SD). The difference was statistically significant ($P \leq 0.001$; Mann-Whitney U test).

the mitochondrion stayed polarized before the rapid permeability increase (Fig. 4 *B*). Interestingly, in the presence of CsA the short-lasting transient voltage drops of lower amplitude were almost completely abolished. This marked protective effect of CsA strongly favors the notion that the rapid depolarizations were caused by openings of the MTP. The duration and, because of the relatively slow dye response time, the amplitude of the voltage drops, varied depending on either the duration of the MTP opening or the (sub-) conductance level of the pore involved. The inhibitory action of CsA, however, was eventually overcome by the MTP-inducing stimulus. In addition, the tripeptide glutathion, which has been shown to prevent MTP opening presumably by maintaining matrix facing sulfhydryl groups in the reduced state (Beatrice et al., 1984), was found to be even more effective in preventing the voltage decay. Despite the presence of an elaborate GSH transport system in mitochondria (Mårtensson et al., 1990) the relatively low rate of matrix GSH supply might limit the protection conferred by this compound. Oxidation of thiols of vicinal cysteinyl residues and the resulting cross-linking of inner membrane proteins has been implicated in the MTP activation mechanism (Fagian et al., 1990). The CsA and GSH effect were slightly additive, indicating that these drugs were acting on different sites within the signal cascade leading to the permeability transition. Taking the experiments shown in Figs. 3 and 4 together it is most likely that in our experimental system light-generated ROS attacked vicinal thiols, possibly leading to protein aggregation and subsequent MTP gating even in the absence of elevated $[Ca^{2+}]$.

The data presented thus far show that during long-lasting recordings of the voltage-sensitive TMRE fluorescence in isolated mitochondria averaged $\Delta\Psi_m$ declined gradually with time. A decrease in membrane potential, however, would rapidly stimulate H^+ extrusion by the H^+ pumps of the respiratory chain in order to maintain $\Delta\Psi_m$. For a net decrease in $\Delta\Psi_m$ to occur the rate of depolarization has to exceed the compensatory H^+ flux. Fig. 1 shows a depolarization when mitochondria were challenged with ADP. The drop in membrane voltage resulted from electrogenic ATP/ADP exchange (Brustovetsky et al., 1996) and the increased rate of H^+ backflux through the F_0F_1-ATPase (Mitchell, 1966; Nicholls, 1982). The rate of the spontaneous depolarization measured in the single organelle, however, was faster than the ADP-induced voltage changes. This might suggest the involvement of ion channels rather than slower carrier-mediated transport processes. Electrophysiological measurements have identified a variety of channel activities in the inner mitochondrial membrane (Mannella, 1992; Tedeschi and Kinnally, 1994). The inhibitory effect of CsA, however, rendered the MTP the most likely candidate. Further support for this hypothesis comes from the experiment shown in Fig. 5, demonstrating that the $\Delta\Psi_m$ transition in a single mitochondrion was associated with the loss of large fluorescent molecules (calcein, ≈620 Da) entrapped in the matrix compartment. The figure depicts the simultaneous recording of the TMRE fluorescence and the fluorescence

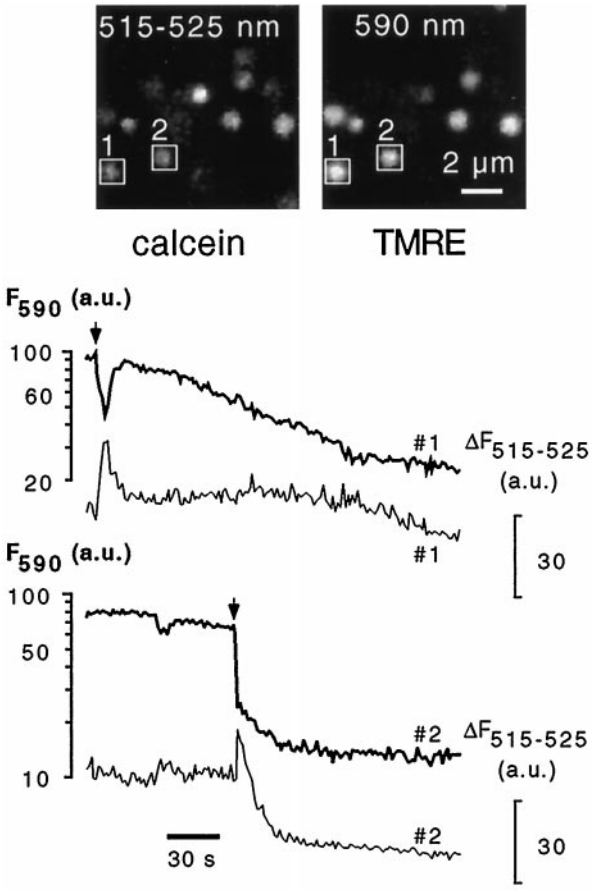

FIGURE 5 Rapid $\Delta\Psi_m$ loss is associated with pore opening. The fluorescent probe calcein (≈620 Da) was introduced into the matrix compartment by exposure of mitochondria to the membrane-permeant acetoxymethyl ester form (calcein/AM). Fluorescence at 590 nm (TMRE, *bold line*) and between 515–525 nm (calcein, *thin line*) was recorded simultaneously in two neighboring mitochondria. In mitochondrion #1 a short-lasting potential drop was visible in the TMRE fluorescence (*1st arrow*) accompanied by transient increase in calcein fluorescence caused by calcein dequenching. Sudden, long-lasting (or irreversible) voltage drops (*2nd arrow*, mitochondrion #2) resulted in an initial increase in the calcein fluorescence (indicating dequenching) and the subsequent irreversible decrease in the calcein signal to background levels caused by loss of dye from the matrix compartment. The slowly decaying second component of the F_{590} signal associated with pore transition was probably caused by contamination from the calcein fluorescence.

originating from calcein (F_{calc}) within the matrix in two individual mitochondria. Mitochondrion #1 displayed a rapid transient depolarization that was mirrored by a F_{calc} transient with similar kinetics. $\Delta\Psi_m$ subsequently decayed gradually with no further change in F_{calc}. Mitochondrion #2 remained stable for nearly 2 min before $\Delta\Psi_m$ rapidly dropped. Most strikingly, this transition was accompanied by a rapid increase in F_{calc} followed by a slower irreversible decrease of F_{calc} to background levels, indicating efflux and permanent loss of the dye. The rapid large amplitude voltage drops therefore coincided with an increase in the permeability of the inner mitochondrial membrane that allows efflux of large molecules. It is possible that the permeability increase underlying the transient depolarization in mito-

chondrion #1 also involved a pathway permeable for calcein. If the pore opening was short in duration the resulting efflux of the dye might have been too small to be resolved in the fluorescence trace. The rapid increase in F_{calc} with the transient decrease in $\Delta\Psi_m$ or immediately following the large amplitude $\Delta\Psi_m$ drop might be explained by a rapid H^+ influx that is associated with the pore opening. The changes in ΔpH, however, are expected to be small under our experimental conditions (0.5 mM KH_2PO_4). In addition, the calcein fluorescence shows only a slight pH-dependence in the physiological range. An alternative explanation has been presented recently by Petronilli and co-workers (1997) showing that the calcein fluorescence emission is significantly quenched by high tetramethylrhodamine concentrations. We therefore tested for a possible interference between the two fluorescence probes under conditions comparable to our experiments. Fig. 6 summarizes the effects of TMRE on the calcein fluorescence emission in aqueous solutions. The calcein concentration in the mitochondrial matrix following our loading protocol has been estimated to be 10–50 μM. We used 400 μM TMRE, which corresponds to $\Delta\Psi_m \approx -200$ mV in the presence of 0.2 μM TMRE in the extramitochondrial buffer solution. This value is close to the average membrane potential reported for state-3 respiring cardiac mitochondria (Lötscher et al., 1980). While TMRE alone did not result in any appreciable fluorescence at 515–525 nm (calcein channel), a significant "spill-over" of the calcein fluorescence into the TMRE channel (590 nm) was detected. The interference with the $\Delta\Psi_m$ measurements in our study was tolerable because MTP recordings were similar in the absence (Figs. 1–4) and presence (Fig. 5) of calcein. After addition of 400 μM TMRE, the calcein fluorescence at 515–525 nm decreased by ~60%. From these data it is concluded that the transient $F_{515-525}$ increase shown in Fig. 5 (*top panel*) was most likely caused by the transient dequenching of the calcein fluorescence during the transient voltage or $[TMRE]_{matrix}$ drop. Because of the higher mobility of TMRE compared to calcein, prolonged pore opening results in a rapid reduction of $[TMRE]_{matrix}$ followed by a slow efflux of calcein. As a result, dequenching of the calcein emission preceded the fluorescence decrease caused by calcein efflux from the mitochondrial matrix.

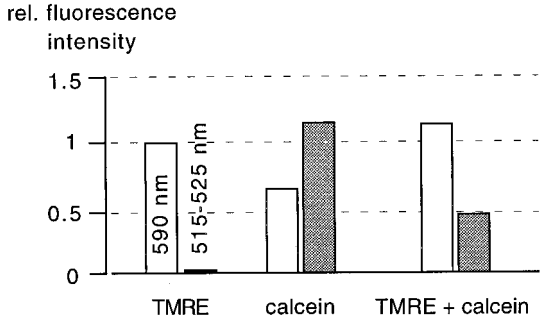

FIGURE 6 Quenching of calcein fluorescence by TMRE. The fluorescence intensity of aqueous solutions of TMRE (400 μM), calcein (35 μM), and TMRE (400 μM) + calcein (35 μM) has been measured on the stage of the confocal microscope with the same settings used for the TMRE/calcein colabeling experiments. For comparison, the pixel intensities have been normalized to the fluorescence intensity of the TMRE solution at 590 nm (*1st bar*). The calcein fluorescence revealed a significant "spill over" into the TMRE channel (590 nm). Furthermore, in the presence of 400 μM TMRE, the calcein fluorescence emission was reduced by ~60%.

DISCUSSION

Combining isolated mitochondria with high-resolution confocal imaging we present a novel approach for studying mitochondrial function. This approach allows us to record fluorescence signals originating from single intact organelles in an experimentally controlled environment. In this first set of experiments, we have used a potentiometric fluorescent probe, TMRE, to resolve electrical events related to the mitochondrial permeability pore transition in individual mitochondria. Prolonged illumination of the mitochondria resulted in the gradual loss of membrane potential measured by averaging over small mitochondrial populations. The protective action of radical scavengers revealed that the voltage decay was caused by the light-induced generation of ROS in the absence of elevated $[Ca^{2+}]$. Catalase was found most effectively to prevent pore transition, suggesting that H_2O_2 and/or H_2O_2-derived radicals were involved. The role of ROS in the mechanism of the mitochondrial permeability pore transition is currently a matter of controversy. Vercesi's group demonstrated in liver mitochondria that catalase exerts marked protection against membrane permeabilization by Ca^{2+} plus prooxidant (Valle et al., 1993; Kowaltowski et al., 1996). In their experiments further protection was conferred by thiol-reducing agents, suggesting protein thiols as primary targets for ROS-mediated oxidation. Furthermore, pore transition and subsequent matrix swelling in response to uncoupling or P_i addition was prevented in the absence of oxygen (Kowaltowski et al., 1996). Based on these findings these authors suggested a crucial role of ROS in the formation/opening of MTP. In their model, besides directly affecting the pore, matrix Ca^{2+} promotes MTP formation by stimulating ROS formation by the respiratory chain and/or inducing conformational changes in membrane proteins that result in exposure of critical cysteine residues. In sharp contrast, using different protocols, Scorrano et al. (1997) and Krasnikov et al. (1997) reported MTP induction under anaerobic conditions. Moreover, photogenerated superoxide anions have been reported to inhibit MTP rather than induce pore opening (Salet et al., 1997). The experiments presented in this paper support the notion that ROS can induce MTP in the absence of Ca^{2+}.

MTP openings induced by ROS in our experimental system were suppressed by thiol reducing agents, e.g., reduced glutathion or dithiothreitol (not shown). Therefore, oxidation of vicinal thiols appears to be a crucial step in MTP formation and/or gating under the conditions used in

this study. Extensive thiol oxidation of membrane proteins might eventually lead to formation of protein aggregates (Fagian et al., 1990). A similar cross-linking of membrane proteins upon irradiation has been demonstrated in fluorescently labeled erythrocyte cell membranes (Sheetz and Koppel, 1979). It has been suggested that the reversibility of MTP might be directly correlated to the extent of protein aggregation (Castilho et al., 1996). The reversible gating of MTP described in this paper therefore suggests that the degree of protein aggregation was likely to be low.

On the level of a single organelle the permeabilization was evident as a rapid depolarization coinciding with a permeability increase for matrix-entrapped calcein. The combined use of a potentiometric tetramethylrhodamine methyl ester (TMRM) and calcein to study the mitochondrial pore transition has recently been introduced by Nieminen and co-workers (1995). In their study calcein was introduced preferentially into the cytosolic compartment of isolated hepatocytes. Mitochondria were identified by their bright TMRM fluorescence. Under control conditions mitochondria excluded calcein. Pore transition induced by incubation with a prooxidant was identified by a redistribution of calcein into the mitochondrial compartment and the concomitant loss of TMRM fluorescence. This approach was recently questioned by the finding that TMRM quenches the calcein fluorescence in aqueous solution and in mitochondria (Petronilli et al., 1997). Therefore, we have studied possible dye interactions between TMRE and calcein in solution under our experimental conditions. TMRE in concentrations expected to be present in charged mitochondria markedly reduced the green calcein emission (515–525 nm). Thus, detection of mitochondrial calcein fluorescence is impaired by the simultaneous presence of TMRE (or TMRM). However, mitochondrial calcein fluorescence in our colabeling experiments was sufficiently high to be detected. As a result of the different redistribution kinetics with which the dyes responded to MTP opening, rapid loss of matrix TMRE caused dequenching of calcein resulting in an increase of F_{calc} that mirrored the time course of F_{TMRE}. This recording artifact did not prevent the accurate resolution of the irreversible loss of matrix calcein associated with prolonged pore openings (see Fig. 5). Probing MTP by calcein entry into the mitochondria in the simultaneous presence of a potentiometric rhodamine dye (Nieminen et al., 1995), however, has several shortcomings. For calcein entry to be detected either large amounts of the dye must enter the matrix or mitochondria must stay constantly depolarized. Short-lasting reversible pore openings are not likely to be resolved using this approach.

Based on our observation that rapid loss of mitochondrial TMRE fluorescence coincided with the efflux of matrix-entrapped calcein together with the protective effect exerted by CsA, it is concluded that the sharp voltage drops in the single mitochondrion resulted from opening(s) of the mitochondrial permeability transition pore. Most strikingly, repetitive gating of MTP between the open and closed state gave rise to marked oscillations of $\Delta\Psi_m$. In addition to the rapid large amplitude voltage transients, drops of $\Delta\Psi_m$ of smaller amplitude (Figs. 1 and 2) were observed. These transient events were reminiscent of the occasional depolarizations in single mitochondria in intact neuroblastoma cells reported by Loew and co-workers (1993) using the same fluorescent dye. The observation that the small transient voltage drops were less likely to occur in the presence of CsA supports the idea that these events were also caused by openings of the transition pore. Thus, the permeability pore transition in the intact organelle subjected to oxidative stress is not a "one-time event." The transition pore opens and closes repetitively causing fast membrane potential fluctuations. With a single channel conductance between 0.6 and 1.0 nS (Zoratti and Szabo, 1995) opening of a single pore for <5 ms would be sufficient to completely dissipate $\Delta\Psi_m$. The limited time resolution provided by the redistribution indicator used in this study prevents the accurate resolution of these fast depolarizations. Moreover, the recharging of the inner membrane by the activity of the proton pumps of the respiratory chain is much slower than the breakdown of $\Delta\Psi_m$ upon MTP opening. Therefore, only pore closures of sufficient duration to allow for the buildup of $\Delta\Psi_m$ and the concomitant redistribution of the indicator can be resolved in the TMRE recordings. Fast fluctuations between multiple open and closed states like those observed in voltage-clamped mitoplast membranes (Szabo and Zoratti, 1992; Zoratti and Szabo, 1995) would result only in a drop in the TMRE fluorescence without any appreciable recovery during short pore closures. Thus, while short openings of MTP are readily detected, short-lasting closures and/or "flickering" of the pore between open and closed states cannot be resolved. Furthermore, the observation of repetitive cycles of discharging and recharging of the inner membrane suggests that in these organelles only one or very few pores had been induced. After prolonged exposure to laser illumination, however, the voltage decay was irreversible. From our experiments it is not clear whether this was due to the continuous production of protein thiol cross-linking or possibly additional lipid peroxidation.

Our experiments provide evidence that elevated matrix Ca^{2+} concentrations are not essential for MTP gating under our experimental conditions. Moreover, no significant depolarization preceding the rapid voltage drop in the single organelle was observed. There is, however, a large body of evidence that matrix Ca^{2+} overload presents a very potent trigger of MTP opening in vitro and in vivo (Zoratti and Szabo, 1995). It can be speculated that repetitive openings and closings of the pore are a general feature of MTP in the intact organelle. It is therefore conceivable that transient openings of MTP caused by Ca^{2+} overload provide a pathway for rapid Ca^{2+} release, thereby removing or at least reducing the pore-inducing stimulus (Bernardi and Petronilli, 1996; Ichas et al., 1997). Thus, pore opening(s) in vivo might present a mechanism protecting mitochondria from prolonged episodes of Ca^{2+} overload and its deleterious effects.

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