Transient and Long-Lasting Openings of the Mitochondrial Permeability Transition Pore Can Be Monitored Directly in Intact Cells by Changes in Mitochondrial Calcein Fluorescence

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ABSTRACT The occurrence and the mode of opening of the mitochondrial permeability transition pore (MTP) were investigated directly in intact cells by monitoring the fluorescence of mitochondrial entrapped calcein. When MH1C1 cells and hepatocytes were loaded with calcein AM, calcein was also present within mitochondria, because (i) its mitochondrial signal was quenched by the addition of tetramethylrhodamine methyl ester and (ii) calcein-loaded mitochondria could be visualized after digitonin permeabilization. Under the latter condition, the addition of Ca^{2+} induced a prompt and massive release of the accumulated calcein, which was prevented by CsA, indicating that calcein release could, in principle, probe MTP opening in intact cells as well. To study this process, we developed a procedure by which the cytosolic calcein signal was quenched by Co^{2+}. In hepatocytes and MH1C1 cells coloaded with Co^{2+} and calcein AM, treatment with MTP inducers caused a rapid, though limited, decrease in mitochondrial calcein fluorescence, which was significantly reduced by CsA. We also observed a constant and spontaneous decrease in mitochondrial calcein fluorescence, which was completely prevented by CsA. Thus MTP likely fluctuates rapidly between open and closed states in intact cells.

INTRODUCTION

In isolated mitochondria, conditions have been described that cause a Ca^{2+}-dependent increase in mitochondrial permeability to ions and solutes with molecular masses up to 1500 Da, matrix swelling, and uncoupling of oxidative phosphorylation. This phenomenon, which has been defined as permeability transition (Hunter and Haworth, 1979), is today ascribed to the opening of the mitochondrial transition pore (MTP), a regulated channel inhibited by submicromolar concentrations of cyclosporin A (CsA) (see Zoratti and Szabo, 1995). The pore appears to coincide with the mitochondrial megachannel, a high-conductance channel identified by patch-clamp studies of rat liver mitoplasts (Bernardi, 1992; Szabo et al., 1992; Szabo and Zoratti, 1992).

The MTP might also serve the physiological function of providing mitochondria with a fast pathway for Ca^{2+} release (Bernardi and Petronilli, 1996; Gunter et al., 1994; Ichas et al., 1994, 1997). Indirect support for this concept was obtained by Altschuld et al. (1992), who described a doubling of $^{45}Ca^{2+}$ retention by cardiomyocytes incubated with CsA, and it has been considerably reinforced by recent results obtained in intact cells, suggesting that pore flickering may amplify IP_3-dependent Ca^{2+} signals arising from the endoplasmic reticulum (Ichas et al., 1997).

Despite the great interest in the mitochondrial permeability transition as a potential effector of cell death (Imberti et al., 1993; Pastorino et al., 1993; Kroemer et al., 1995, 1997), evidence for its occurrence in vivo remains indirect and is largely based either on secondary events, such as mitochondrial depolarization, or on pharmacological tools, such as the effects of in vitro pore inducers or inhibitors (like CsA). The shortcomings are obvious, because depolarization can be caused by a variety of events (most notably, increased ATP demand), whereas CsA interferes with calcineurin-dependent signaling, which like MTP has a prominent Ca^{2+} dependence (Bennett and Norman, 1986). In addition, the indirect approaches used so far do not allow us to address the cellular modulation of MTP, leaving crucial issues, such as the cause-effect relationship between pore opening and $\Delta\psi_m$ decrease, unsolved. These considerations explain the intrinsic interest in developing an unequivocal tool to measure MTP opening in living cells.

Here we developed a method to assess MTP opening in calcein-AM-loaded cells through Co^{2+} quenching of cytosolic calcein fluorescence. Mitochondrial fluorescence of calcein shows an abrupt decrease induced by MTP agonists and a gradual decrease in the absence of any treatment, which are both inhibited by CsA. These results are consistent with two modes of MTP opening, i.e., transient and long-lasting. Preliminary accounts of these results have been published in abstract form (Petronilli et al., 1997, 1998).

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MATERIALS AND METHODS

Cell isolation and culture

Rat liver parenchymal cells (hepatocytes) were isolated by collagenase perfusion according to the method of Seglen (1976), with minor modifications (Venerando et al., 1994). Yields of 3–4 $\times$ 10^8 cells/liver with

90–95% viability by trypan blue exclusion were routinely obtained. After isolation cells were plated onto 22-mm-diameter round coverslips precoated with rat tail collagen, and incubated in Williams' medium E supplemented with 10 IU/ml penicillin, 10 μg/ml streptomycin, 0.005 μg/ml gentamycin, 0.02 units/ml insulin, and 10% heat-inactivated fetal calf serum, at 37°C in an atmosphere of 5% CO_2/95% air.

MH1C1 rat hepatoma cells were seeded on uncoated 22-mm-diameter round glass coverslips and grown in Ham's F-10 nutrient mixture supplemented with 20% fetal calf serum, at 37°C in an atmosphere of 5% CO_2/95% air.

Mitochondrial isolation and calcein loading

Rat liver mitochondria were prepared by standard differential centrifugation as described previously (Costantini et al., 1995). Mitochondrial calcein loading was performed by incubating a mitochondrial suspension (20 mg/ml, in a buffer containing 250 mM sucrose, 10 mM Tris/3-(*N*-morpholino)propanesulfonic acid, 0.1 mM EGTA, pH 7.4) with 2 μM calcein AM. After 15 min of incubation at 25°C, mitochondria were centrifuged at 12,000 $\times$ *g* for 2 min at 4°C, washed with ice-cold medium without calcein, and finally resuspended in the same medium at a final concentration of 80 mg mitochondrial protein/ml.

Spectrofluorometry

Spectrofluorometric measurements were performed in a Perkin-Elmer LS5 fluorometer equipped with a thermostatted cuvette mount and magnetic stirring. Fluorescence emission was monitored at 25°C, using the indicated excitation and emission wavelengths with slits of 2 and 5 nm, respectively. To study the interactions between calcein and TMRM within mitochondria, calcein-AM-loaded rat liver mitochondria (0.25 mg mitochondrial proteins/ml) were incubated in a medium containing 0.25 M sucrose, 5 mM succinate, 2 μM rotenone, 1 mM P_i, 20 μM EGTA, 20 mM Tris-3-(*N*-morpholino)propanesulfonic acid (pH 7.4), and when required, 0.5 μM TMRM was added.

Cell microinjection

Calcein microinjection into hepatocytes was performed essentially according to the method of Rutter et al. (1996). Eppendorf sterile fentotips were pulled to give an external tip diameter of 0.2–0.5 μm. An Eppendorf 5171 micromanipulator and 5424 pressure injector with a Zeiss Axiovert 100 TV epifluorescence microscope and 32×, 0.5 NA objective, were used. Calcein free acid (0.25 mg/ml) was microinjected into the cell cytoplasm at a pressure between 500 and 2000 hPa during an injection period of 0.2–1.0 s.

Calcein staining procedures

Cells were loaded for 15 min with 1 μM calcein AM at 37°C in a modified Hanks' balanced solution (Sigma), which was devoid of phenol red and $NaHCO_3$ and supplemented with 10 mM Na-HEPES (pH 7.3) and then maintained in the modified Hanks' balanced solution. When quenching of cytosolic and nuclear calcein fluorescence was required, 1 mM $CoCl_2$ was present during calcein loading. The extent of quenching was affected by the length of time cells spent in culture before experiment. In fact, quenching was more pronounced when cells were cultured for 2 days rather than 1 day. After attainment of quenching, cells were washed free of calcein and Co^{2+}, and experimental recordings began after the coverslips were transferred into an open chamber that was mounted on the stage of an inverted microscope connected to either a laser or a xenon light source. The extent of calcein loading can be affected by the activity of the multidrug resistance (MDR) P-glycoprotein (Hollo et al., 1994). Therefore, CsA and cyclosporin H (CsH), which both inhibit MDR (Dietel et al., 1994), were added to the cells after calcein and Co^{2+} loading and 30 min before experimental recording.

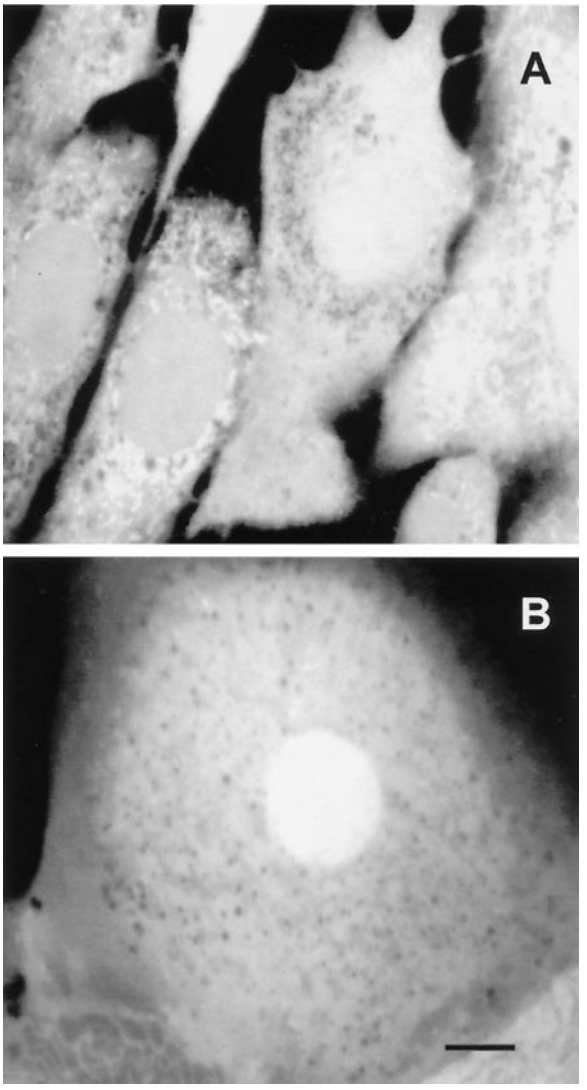


FIGURE 1 Confocal image of MH1C1 cells (*A*) and one hepatocyte (*B*) loaded with 1 μM calcein-AM. Both cell types showed a diffuse fluorescence pattern with rare voids that differ from mitochondria for size, number, and distribution. Bar, 7 μm.

Cell permeabilization

Cells were permeabilized with 10 μM digitonin for 10 min in a cytosolic buffer containing 0.1 M KCl, 1 mM P_i, 1 mM $MgCl_2$, 50 μM EGTA, and 10 mM K-HEPES (pH 7.3). Succinate (5 mM) was used as the oxidizable substrate.

Epifluorescence and laser confocal microscopy

An inverted microscope (Olympus IMT-2) was equipped for epifluorescent illumination and included a xenon light source (75 W), a 12-bit digital cooled CCD camera (Micromax; Princeton Instruments), and appropriate excitation and emission cubes. Fluorescent cell images obtained with 40×, 1.3 NA and 100×, 1.3 NA oil immersion objectives (Nikon) were collected over exposure times ranging from 100 to 500 ms. Data were acquired and analyzed with Metamorph software (Universal Imaging). In the case of confocal studies, the same objectives were used in a Nikon Diaphot-300, which was part of the real-time confocal system (RCM 8000; Nikon). Excitation wavelength/detection filter settings for each of the fluorescent

dyes were as follows: calcein, 488/525 ± 25 nm bandpass; tetramethylrhodamine methyl ester (TMRM) and propidium iodide, 568/585 longpass. In some experiments calcein and TMRM or propidium iodide fluorescence emissions were collected simultaneously by using two separate color channels on the detector assembly (green and red planes of the resulting RGB images, respectively). Sequential 512 × 484 pixel color (8 bits per pixel per plane) confocal images were acquired and stored typically at 30-s intervals.

Data analysis

Using the data acquisition software, we identified clusters of several mitochondria (10–20) as regions of interest (ROIs), and background was identified as an area without cells. Subsequently, in most of the experiments sequential digital images were obtained every 30 s for 30–60 min, and the average fluorescence intensity for all of the ROIs and background was recorded for subsequent analysis. Calcein fluorescence intensity was analyzed as the changes in both individual ROIs and all of the ROIs, minus background, for each coverslip. Because the initial values of fluorescence intensities were different in the various ROIs, average fluorescence is reported as the percentage of the initial values.

RESULTS

Mitochondrial accumulation of calcein

Primary cultures of rat hepatocytes or MH1C1 rat hepatoma cells were loaded with a wide range of calcein AM concentrations (0.1–2 μM). Both cell types displayed a diffuse pattern of cytosolic fluorescence, irrespective of the duration of the loading procedure (Fig. 1). In our hands, a small number of cells showed rare fluorescence voids, which differed from mitochondria for size, number, and distribution. Similar voids were also obtained in cells loaded with other dyes, such as the fluorescein derivative BCECF AM (results not shown). These images, however, cannot reveal whether mitochondria contain calcein. Even assuming that rapid cytosolic deesterification of calcein AM prevents filling of mitochondria with calcein AM (followed by hydrolysis and probe entrapment), it appears unlikely that mitochondria can be detected, given the bright cytosolic signal. Furthermore, in confocal microscopy, fluorescence spreading magnifies the size of emitting objects, while reducing that of dark spots.

This problem was experimentally addressed by cell microinjection with unesterified calcein, which is not permeable to mitochondria (results not shown). Fig. 2 *A* shows that the fluorescence pattern obtained in these protocols mimicked that observed in calcein-AM-loaded cells, including the rare fluorescence voids that unambiguously differed from the distribution of mitochondria, as judged from TMRM coloading (Fig. 2 *B*).

These results cannot rule out the possibility that mitochondria were in fact filled with calcein when cells were loaded with calcein AM. In principle, this can be assessed by selective quenching of the mitochondrial calcein signal. With this aim in mind, we studied the in vitro interaction between calcein and TMRM, which is electrophoresed into the mitochondrial matrix by $\Delta\psi_m$ (Ehrenberg et al., 1988). Fig. 3 *A* shows that the fluorescence emission of 50 μM calcein in aqueous solution was totally quenched by equimolar amounts of TMRM. Under these conditions the emission peak of TMRM increased, suggesting that an energy transfer process was taking place, possibly reflecting complex formation (Fig. 3 *B*). Fig. 3 *C* documents that a similar interaction could be observed in rat liver mitochondria loaded with calcein AM. Indeed, calcein emission was decreased by TMRM addition, and it was restored by uncoupling agents that cause TMRM release from mitochondria. On the other hand, Fig. 3 *D* shows that calcein did not affect the typical fluorescence variations that can be observed by monitoring TMRM accumulation and release at longer wavelengths. In the absence of calcein, the excitation-emission couple used to monitor calcein fluorescence did not show any change when mitochondria were incubated with TMRM (not shown). Finally, when mitochondria were incubated with the unesterified form of calcein, which is not membrane permeant, the fluorescence emission was not modified by TMRM addition (results not shown), indicating that calcein quenching requires TMRM in its immediate vicinity, i.e., in the same cellular compartment.

To study whether calcein and TMRM also interact in mitochondria within intact cells, cultured hepatoma cells were loaded with 1 μM calcein AM and then exposed to increasing concentrations of TMRM. The images in the left column of Fig. 4 show the confocal images obtained by attributing a green color to calcein and a red color to TMRM (*A* and *C*, respectively). Note that yellow areas indicate

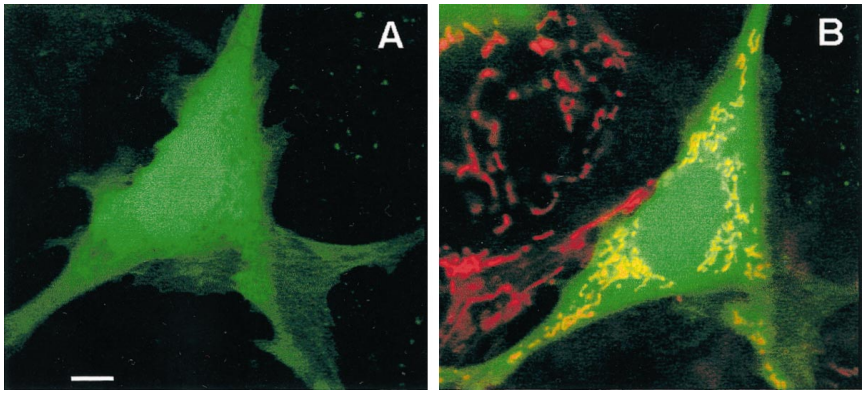


FIGURE 2 Confocal image of a single hepatocyte injected with calcein free acid (*A*) and then loaded with TMRM (*B*). The green and red components of the images indicate calcein and TMRM fluorescence, respectively. Dark round voids in calcein fluorescence images could be detected, which did not correspond to mitochondria, as indicated by the lack of filling with TMRM. Note that after TMRM had been accumulated, no additional dark voids appeared in the calcein signal. Bar, 7 μm.

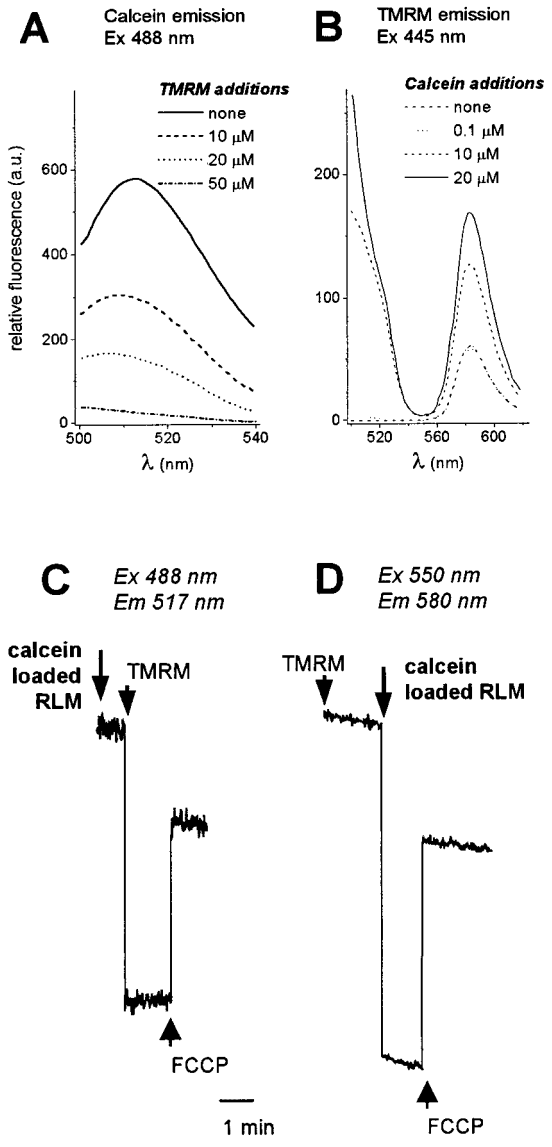


FIGURE 3 Interactions between calcein and TMRM in aqueous solution and in mitochondria. The fluorescence emission of an aqueous calcein solution (50 μM) was totally quenched by equimolar amounts of TMRM (*A*), whereas the emission peak of TMRM was increased (*B*). The fluorescence signal of energized mitochondria loaded with calcein AM (see Materials and Methods) was quenched by the uptake of TMRM and returned close to baseline values upon the release of TMRM induced by depolarization with FCCP (*C*). Calcein-loaded mitochondria were able to take up TMRM and release it upon depolarization (*D*). RLM, rat liver mitochondria.

colocalization of the red and green signals. The images in the right column show calcein fluorescence only, as obtained by subtracting the red component from the images shown on the left (Fig. 4, *B* and *D*, respectively). TMRM at low concentration (0.1 μM) did not modify the diffuse cellular calcein staining (Fig. 4, *A* and *B*), which was observed in the absence of TMRM (e.g., Fig. 1). When the concentration of TMRM was increased to 3 μM, on the other hand, calcein fluorescence displayed a different pattern, with dark holes corresponding to mitochondria, as clearly documented by comparing *C* and *D* in Fig. 4. Thus it appears that voids in calcein fluorescence can be generated by its interaction with TMRM within mitochondria.

MTP-dependent mitochondrial release of calcein in permeabilized cells

To test whether calcein was trapped inside mitochondria, primary cultures of hepatocytes were loaded with 1 μM calcein AM, followed by cell permeabilization with digitonin. This procedure revealed the existence of clusters of round fluorescent bodies (Fig. 5). Despite the loss of the typical spindle shape characteristic of mitochondria in situ, these bodies were identified as mitochondria by their ability to accumulate TMRM upon energization (not shown). To test whether intramitochondrial calcein could be released through the MTP, permeabilized cells were energized with succinate both in the absence (Fig. 5, *A* and *B*) and in the presence (Fig. 5, *C* and *D*) of 1 μM CsA. After the baseline images had been taken, 200 μM $CaCl_2$ was added and sequential images were collected at various time intervals. Ca^{2+} loading caused a marked decrease in mitochondrial calcein fluorescence that was substantially prevented by CsA (Fig. 5). Indeed, CsA reduced both the rate and the extent of calcein fluorescence decrease induced by Ca^{2+}, as monitored by plotting the averaged fluorescence intensities versus time (Fig. 5). From this kinetic analysis it appears that the rate of mitochondrial calcein release in permeabilized cells after MTP opening closely matches that observed in isolated mitochondria by means of classical swelling measurements. The results of Fig. 5 thus represent a direct visualization of the consequences of MTP opening and establish an unambiguous assay of MTP in permeabilized cells.

Co^{2+} quenching of cytosolic calcein allows visualization of mitochondria in situ

Because mitochondrial calcein release can visualize MTP opening in single mitochondria, we developed a procedure for selective quenching of the cytosolic calcein signal in intact cells based on the well-known capacity of several metal ions to reduce or abolish calcein emission (Wallach and Steck, 1963). This approach has already been successfully employed in monitoring changes in mitochondrial Ca^{2+} in isolated cardiomyocytes after the cytosolic emission of Indo 1 is quenched with Mn^{2+} (Miyata et al., 1991). Calcein emission is reduced by Mn^{2+} and completely abolished by Co^{2+} and Cu^{2+}. Because Cu^{2+} addition is likely to promote peroxidative processes, which are harmful to the cell and can favor MTP opening, we focused on Co^{2+}, which is not transported by the mitochondrial Ca^{2+} uniporter (Basso et al., manuscript in preparation). When cells were coloaded with calcein AM and $CoCl_2$, calcein fluorescence was quenched in both cytosolic and nuclear

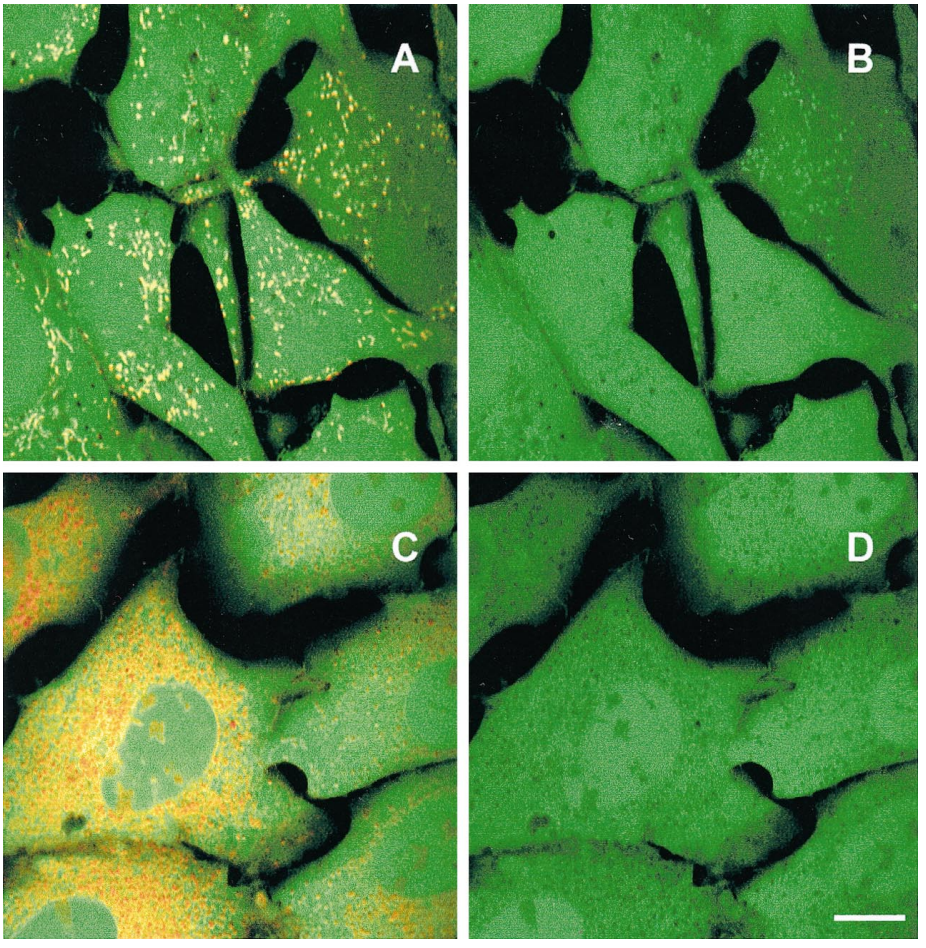


FIGURE 4 Confocal images of MH1C1 cells coloaded with TMRM and calcein AM. The green and red components of the images indicate calcein and TMRM fluorescence, respectively. (*A* and *C*) Both calcein and TMRM fluorescence (*green* and *red*, respectively). (*B* and *D*) Only calcein fluorescence, as obtained by eliminating the red component from the images in *A* and *C*. MH1C1 cells were loaded with 1 μM calcein-AM in the presence of 0.1 μM TMRM (*A* and *B*). A diffuse calcein fluorescence pattern appeared with rare voids. TMRM loading was then increased by adding 3 μM TMRM (*C* and *D*). After accumulation of high concentrations of TMRM, mitochondria assumed a round shape (*red* in *C*), and calcein fluorescence showed a punctate pattern with dark holes corresponding to mitochondria (*D*). Bar, 10 μm.

compartments, resulting in the appearance of glowing bodies against a dark background (Fig. 6).

These images could be obtained with the confocal microscope (Fig. 6 *A*) or with a conventional fluorescence microscope equipped with a cooled CCD camera (Fig. 6 *B*). The latter setup proved to be essential in minimizing the phototoxicity resulting from the intracellular accumulation of fluorescent molecules, which is exacerbated by irradiation with laser sources. The calcein fluorescent bodies visualized after Co^{2+} quenching (Fig. 7 *A*) were identified as mitochondria by the subsequent addition of TMRM (Fig. 7 *B*). Furthermore, and as observed in isolated mitochondria, the mitochondrial fluorescence of calcein was quenched by TMRM, as shown by the antiparallel changes in the two fluorescence emissions induced by FCCP (Fig. 7 *C*).

Probing MTP opening in intact cells

MH1C1 cells loaded with Co^{2+} and calcein AM were imaged after external Co^{2+} was removed. The addition of A23187, a Ca^{2+} ionophore, induced an abrupt decrease in mitochondrial calcein fluorescence (Fig. 8). A similar result (not shown), although to a smaller extent, was obtained by treating cells with *t*-butylhydroperoxide or phenylarsine oxide, which are both well-characterized inducers of MTP opening in isolated mitochondria (Beatrice et al., 1984; Bernardi, 1992). At variance with the complete release of calcein, which is produced by MTP opening in isolated mitochondria and permeabilized cells, intact cells always retained appreciable amounts of calcein (Fig. 8). The possibility of passive membrane binding was addressed by treating Co^{2+} and calcein-AM-coloaded hepatoma cells with 30 μM alamethicin, a pore-forming antibiotic (Latorre and Alvarez, 1981). The occurrence of membrane permeabilization was monitored by nuclear staining with propidium iodide, which is cell impermeant under control conditions (Darzynkiewicz et al., 1982; Gores et al., 1988) (Fig. 9 *A*). Alamethicin pore formation caused a precipitous and complete release of calcein from cells concomitantly with the entry of propidium iodide (Fig. 9 *B*), indicating that mitochondrial calcein retention cannot be explained by passive binding. On the other hand, the decrease in the mitochondrial fluorescence of calcein induced by ionophore addition can be attributed to MTP opening, because it was significantly reduced by CsA. This effect was specific, because CsH, a CsA analog that does not inhibit MTP in isolated mitochondria (Nicolli et al., 1996), was ineffective.

It is of interest that a quantitative analysis of the mitochondrial calcein fluorescence versus time revealed a constant decrease in untreated cells, i.e., without the addition of

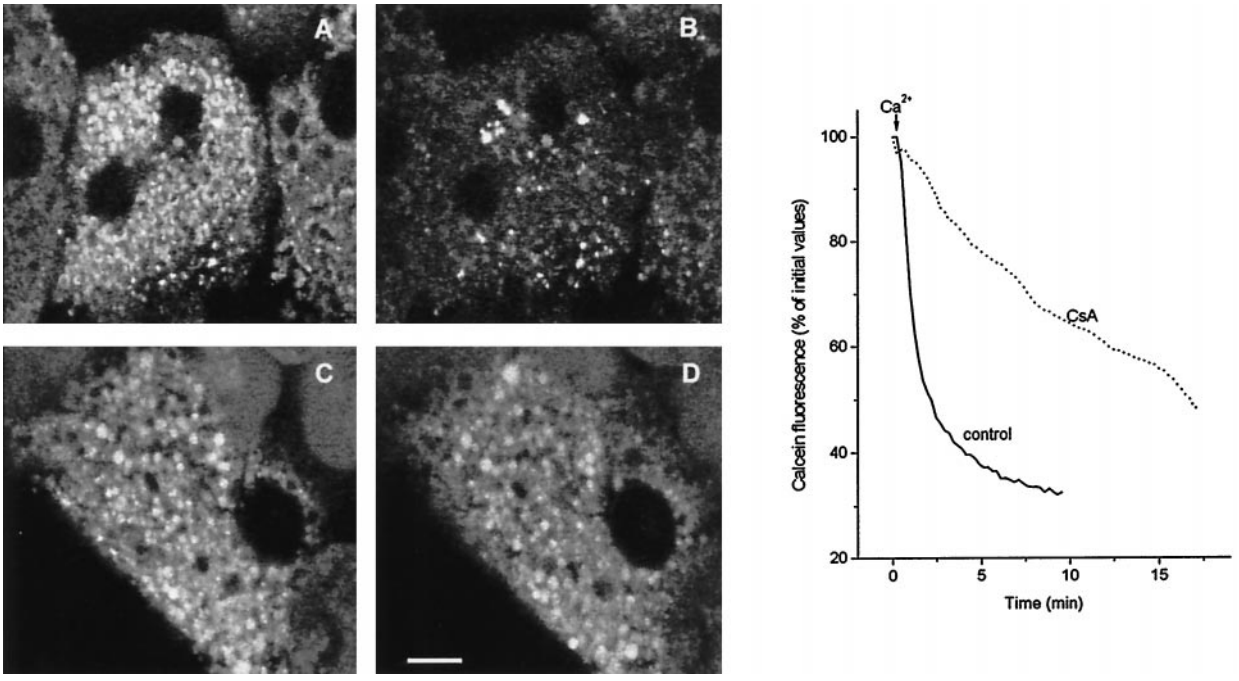


FIGURE 5 Effect of CsA on the release of mitochondrial calcein in permeabilized hepatocytes. Calcein-loaded hepatocytes were permeabilized and energized with succinate (see Materials and Methods). After the baseline confocal images had been collected (*A* and *C*), 200 μM $CaCl_2$ was added, and sequential images were collected at 30-s intervals. In the absence of CsA, Ca^{2+} loading caused a marked decrease in mitochondrial calcein fluorescence, which was almost complete after 3 min (*B*). In the presence of CsA, the decrease in mitochondrial calcein fluorescence after Ca^{2+} loading was negligible (*D*), indicating that the marked fluorescence decrease was caused by calcein exit through MTP. Bar, 10 μm. The plot shows the time course of fluorescence changes induced by Ca^{2+} addition. Data are the mean of 10 ROIs expressed as the percentage of the initial values.

pore inducers. This process was independent of the sampling rate (Fig. 10), ruling out a phototoxic effect. Strikingly, in CsA-treated cells the fluorescence signal of mitochondrial calcein was stable with time and significantly different from that recorded in both untreated and CsH-treated cells (Figs. 8 and 10), suggesting that the MTP may fluctuate between open and closed states in intact cells.

DISCUSSION

The present paper describes a direct method for probing MTP opening at the single-cell level by monitoring the mitochondrial fluorescence of calcein. By using this procedure, we could detect transient and long-lasting openings of MTP in the absence or presence of inducers, respectively.

Strategies for the direct monitoring of MTP

In principle, MTP could be studied directly by following the entry of a molecule that is not able to cross the inner mitochondrial membrane. The spectrum of suitable molecules is limited by the following minimum requirements: (i) molecular mass less than 1.5 kDa; (ii) little or no hydrophobicity to minimize membrane binding; (iii) lack of utilization as a substrate by mitochondrial enzymes.

Among the available fluorescent molecules, calcein has been selected as the probe of choice to detect pore opening with imaging techniques (Nieminen et al., 1995). Calcein has a suitable molecular mass (622 Da) and, despite its aromatic core, is highly hydrophylic because of its six negative and two positive charges at pH $\cong$ 7 (Wallach and Steck, 1963). Because of its negligible membrane binding, it has been used to investigate membrane fusion processes (Kendall and MacDonald, 1982; Perin and MacDonald, 1989; Woodbury and Hall, 1988) as well as membrane transport and intactness (Benachir and Lafleur, 1995; Braut Boucher et al., 1995; Hollo et al., 1994). Although it is cell impermeant, calcein can be easily loaded into cells by using its acetomethyl ester form. Finally, calcein does not undergo Ca^{2+}-dependent changes in fluorescence at physiological pH (Wallach and Steck, 1963), although its emission can be quenched by several metals, including Co^{2+}, at any pH value (Kendall and MacDonald, 1982; Wallach and Steck, 1963).

Mitochondrial calcein as a probe for MTP

Our study reveals that calcein is trapped inside mitochondria after loading of hepatocytes and MH1C1 cells (this paper) and NRK and HeLa cells (not shown) with its acetoxymethyl ester. Indeed: (i) fluorescence voids appeared in calcein-AM-loaded cells upon further addition of TMRM, indicating calcein quenching within mitochondria; (ii) a distinct punctate fluorescence persisted after digitonin treatment of calcein-AM-loaded cells (these fluorescence spots could unequivocally be identified as mitochondria by co-

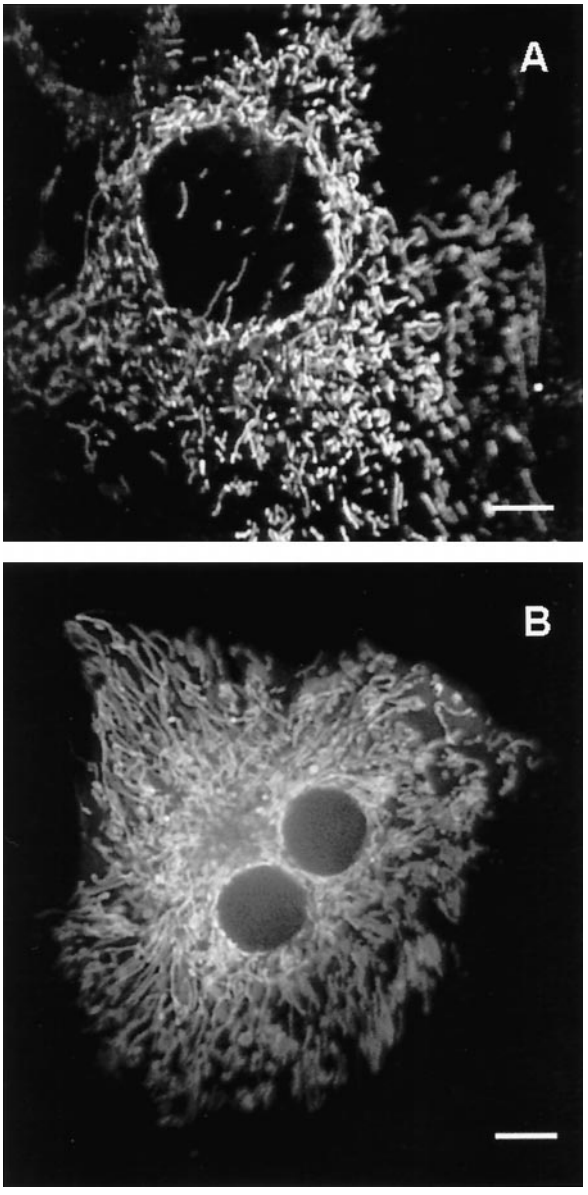


FIGURE 6 Co^{2+} quenching of cytosolic and nuclear calcein fluorescence. MH1C1 cells (*A*) and hepatocytes (*B*) were loaded with calcein-AM in the presence of 1 mM Co^{2+} and visualized by laser confocal (*A*; bar, 6.5 μm) and epifluorescence (*B*; bar, 10 μm) microscopy, respectively. This procedure caused a profound quenching of cytosolic and nuclear fluorescence.

loading of TMRM after permeabilization; not shown); (iii) after Co^{2+}- quenching, the residual fluorescence of calcein colocalized with and was quenched by TMRM (Fig. 7).

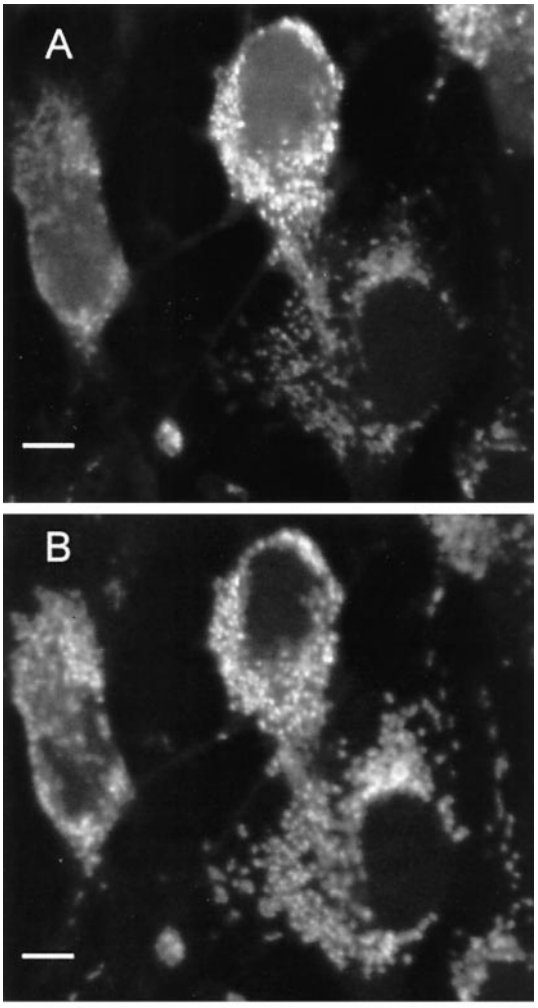
In a previous report (Nieminen et al., 1995) fluorescence voids in the calcein signal were identified as mitochondria by colocalization with TMRM. Treatment with agents known to induce a permeability transition in isolated mitochondria caused the appearance of calcein fluorescence within these voids, suggesting that a permeability transition had occurred in the intact cells. We suspect that intramitochondrial calcein fluorescence is masked when cells are coloaded with TMRM because of fluorescence quenching. In this scenario, the appearance of mitochondrial calcein fluorescence at the onset of depolarization would depend on the release of TMRM, resulting in dequenching of intramitochondrial calcein. In any case, the clear mitochondrial localization of calcein demonstrated here and the quenching of its fluorescence signal by TMRM (Figs. 3, 4, and 7) demand proper controls in dual-labeling experiments before deductions can be made about the status of intramitochondrial calcein, and therefore about MTP opening.

The experiments performed on permeabilized cells showed that Ca^{2+} addition induced a CsA-sensitive loss of calcein fluorescence (Fig. 5), suggesting that calcein release can be used to monitor MTP activity. To detect the changes in mitochondrial calcein fluorescence in intact cells, the cytosolic signal was selectively quenched by Co^{2+}. After this procedure, cells in the absence of any stimuli showed a gradual and spontaneous fluorescence decrease, which is suggestive of transient pore openings under resting conditions (Figs. 8 and 10). On the other hand, treatments known to open the MTP in vitro induced a prompt decline in the mitochondrial fluorescence (Fig. 8). Based on the experimental evidence obtained in permeabilized cells (Fig. 5), calcein release likely contributes to the decrease in mitochondrial fluorescence induced by Ca^{2+} ionophores in Co^{2+}-quenched cells. However, this technique cannot discriminate between calcein release and Co^{2+} entry as the mechanism underlying the spontaneous decrease in the mitochondrial fluorescence of calcein. Nevertheless, the inhibition by CsA and the lack of any significant effects by CsH strongly suggest that the changes in mitochondrial calcein fluorescence reflect MTP openings. Taken together, these data provide a simple, direct assay of mitochondrial permeabilization to solutes that should find widespread applications in the monitoring of mitochondrial function in situ.

Despite its potential, the calcein-Co^{2+} technique presents several shortcomings. Although it is not transported by the Ca^{2+} uniporter (Basso et al., manuscript in preparation), Co^{2+} inhibits Ca^{2+} uptake and slightly decreases respiration. It must be realized, however, that most cytosolic Co^{2+} is bound to calcein, and that the MTP appears to respond normally to its modulators, both after permeabilization and in the intact cells (Figs. 5 and 8, respectively).

MTP opening in situ

An appreciable decrease in the mitochondrial calcein signal could only be observed when cells were treated with Ca^{2+} ionophores, whereas smaller changes were obtained with oxidants, and uncouplers were ineffective (results not shown). Because the MTP is voltage-dependent (Bernardi et al., 1994), the latter result may appear surprising, because one would expect MTP opening upon depolarization in situ. Yet, not only does depolarization require rather high concentrations of matrix Ca^{2+}, a condition that may not be easily achieved in vivo; it also generates conditions (matrix acidification and increased concentrations of ADP and Mg^{2+}) that powerfully inhibit the pore and thus counteract the effect of the voltage drop (Petronilli et al., 1993).



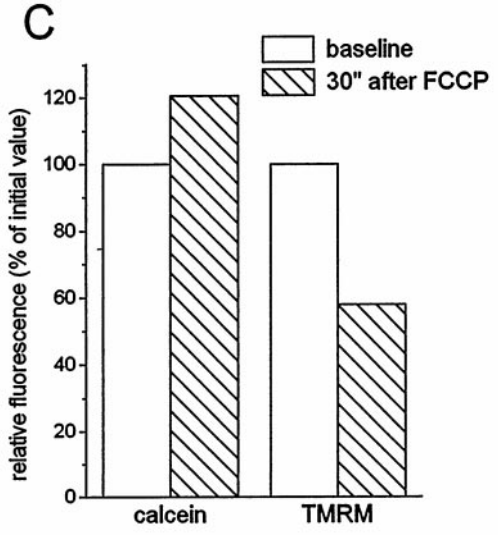


FIGURE 7 Mitochondrial colocalization of calcein and TMRM fluorescence in Co^{2+}-treated cells. MH1C1 cells coloaded with Co^{2+} and calcein-AM were treated with 0.2 μM TMRM and imaged by confocal microscopy. The calcein and TMRM fluorescence components of the image are shown in *A* and *B*, respectively. Bar, 7 μm. (*C*) Fluorescence levels of the two probes before and 1 min after the addition of 2 μM FCCP. The uncoupler treatment caused an increase in mitochondrial calcein fluorescence concomitant with TMRM release (*C*).

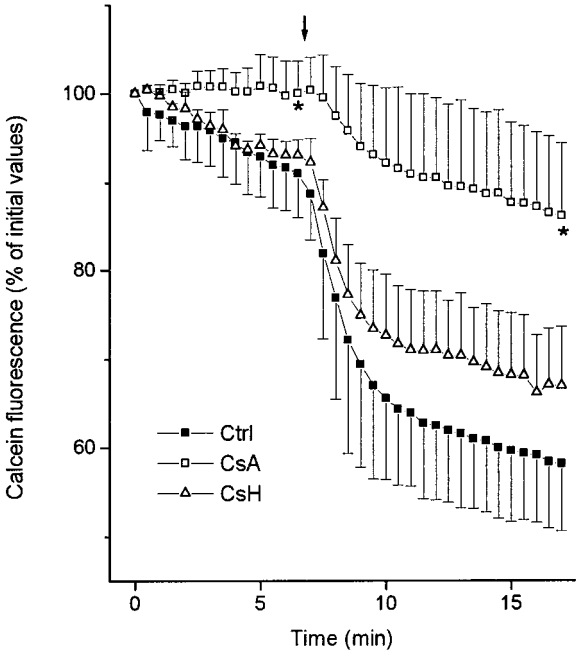


FIGURE 8 Effect of CsA and CsH on the changes of mitochondrial calcein fluorescence in intact cells. MH1C1 cells coloaded with Co^{2+} and calcein AM were imaged, and frames were collected at 30-s intervals in the absence (control, ■) and in the presence of either 1.5 μM CsA (□) or 1.5 μM CsH (△). Where indicated (↓), 2 μM A23187 was added. Mitochondrial fluorescence changes were analyzed as described in Materials and Methods. Values are means ± SD of different experiments (control, $n = 5$; CsA, $n = 6$; CsH, $n = 3$). *, Significantly different from control cells (unpaired *t*-test, $p < 0.01$). Note that before the rapid decrease induced by the Ca^{2+} ionophore, the mitochondrial fluorescence of calcein showed a spontaneous decrease that was significantly prevented by cyclosporin A. Conversely, the values for CsH-treated cells were not significantly different from control values.

Thus, and predictably for the needs of energy conservation, in the intact cell the complex balance between inducers and inhibitors appears to favor the closed pore state, making its prolonged opening an uncommon event. We note that a considerable amount of calcein is retained by mitochondria in intact cells after the large efflux induced by Ca^{2+} ionophores. This finding suggests that in intact cells the extent or the duration of pore opening is limited even after the process has been started, at variance with the complete release observed in isolated mitochondria and permeabilized cells.

Most investigators consider MTP opening in vivo an irreversible event leading to energy dissipation and cell death. Recent results obtained both in intact cells (Ichas et al., 1997) and in single isolated mitochondria (Hüser et al., 1998), however, suggest that the pore opens transiently (i.e., reversibly) and may mediate a fast Ca^{2+} release process. Transient openings of the MTP might thus result in minor changes in $\Delta\psi_m$, which would be without any detrimental effect on cell viability. $\Delta\psi_m$ oscillations have been observed in various cell types (Loew et al., 1993; O'Rourke et al., 1994), and it is tempting to speculate that these are linked to transient MTP openings, although we never detected oscillations in $\Delta\psi_m$, as monitored by TMRM fluorescence (data

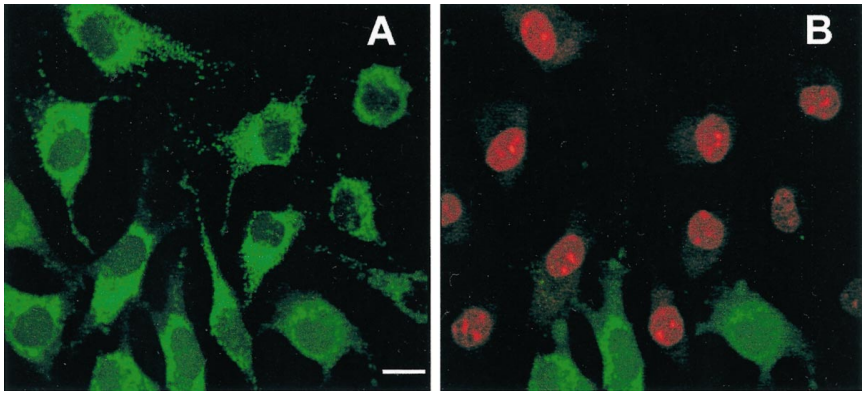


FIGURE 9 Effect of alamethicin on cellular calcein fluorescence. Co^{2+} and calcein-AM coloaded MH1C1 cells were imaged by confocal microscopy (*A*) and then treated with 30 μM alamethicin, a pore-forming antibiotic, and with 1 μM propidium iodide (*B*, 30 min after additions). Alamethicin caused a rapid release of calcein from cells, concomitantly with propidium iodide entry, as shown by the disappearance of the green calcein fluorescence paralleled by red staining of nuclei with propidium iodide. Although this process started at different times in different cells (not shown), once started it was completed in less than 30 s. Bar, 17 μm.

not shown), despite the spontaneous decrease in mitochondrial calcein fluorescence. However, TMRM is not adequate to detect changes in $\Delta\psi_m$ in the millisecond range, which is well above the time required for the open-closed transition of MTP (Szabo and Zoratti, 1991). The fact that TMRM fluorescence does not change over time indicates that the gradual decrease in calcein fluorescence is related to transient MTP activity, because a prolonged or irreversible opening should have resulted in a $\Delta\psi_m$ drop.

The results of the present work thus support the existence of reversible MTP openings of short duration, which are consistent with a physiological role for the permeability transition. Furthermore, they indicate that powerful inhibitory systems prevent long-lasting, potentially irreversible openings, which could be lethal. How these inhibitory systems may be overcome in accidental and programmed cell death remains a key question that the calcein-entrapment technique may help to resolve.

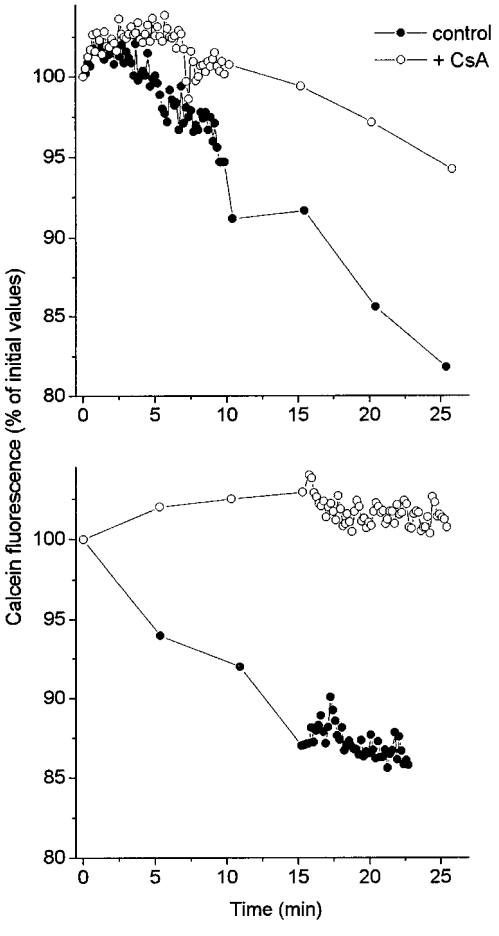


FIGURE 10 Effects of CsA on the spontaneous changes in mitochondrial calcein fluorescence in MH1C1 cells at varying sampling rates. Calcein fluorescence was monitored by using two different sampling protocols. Images were acquired every 10 s for 10 min and every 5 min for 15 min in the fast and slow sequences, respectively, as shown in *A* and *B*. Mitochondrial fluorescence changes were analyzed as described in Materials and Methods. Each data point is the average of three different experiments. ●, Control cells; ○, CsA (1.5 μM)-treated cells. The spontaneous decrease in mitochondrial calcein fluorescence was independent of sampling rates and was prevented by CsA.

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