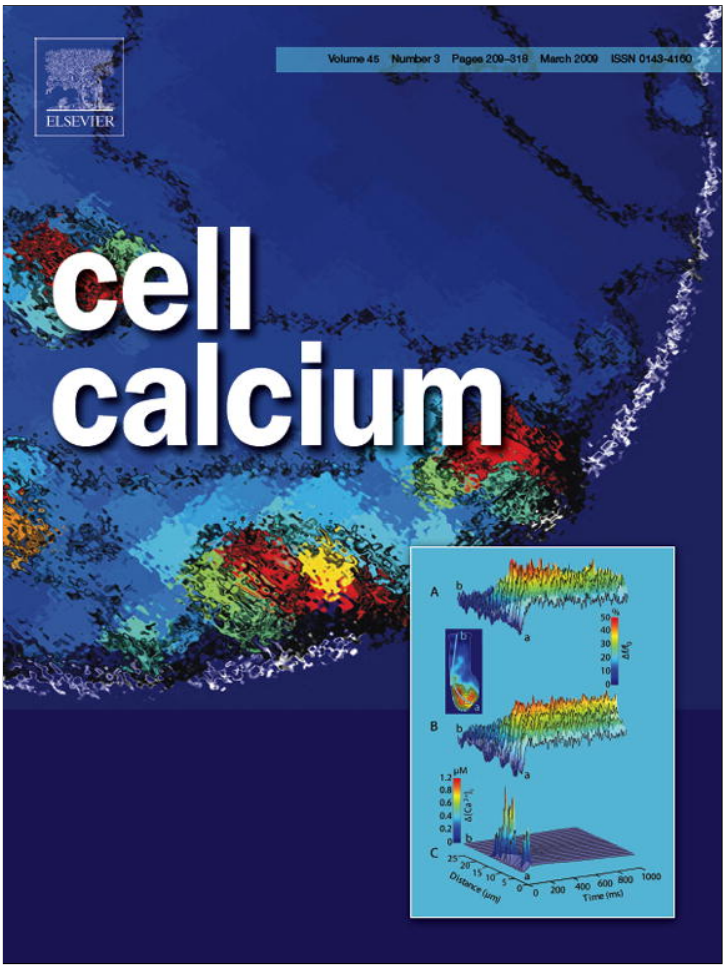

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Mitochondrial free [Ca^{2+}] levels and the permeability transition

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ABSTRACT

Mitochondrial Ca^{2+} activates many processes, from mitochondrial metabolism to opening of the permeability transition pore (PTP) and apoptosis. However, there is considerable controversy regarding the free mitochondrial [Ca^{2+}] ($[Ca^{2+}]_M$) levels that can be attained during cell activation or even in mitochondrial preparations. Studies using fluorescent dyes (rhod-2 or similar), have reported that phosphate precipitation precludes $[Ca^{2+}]_M$ from increasing above 2–3 μM. Instead, using low-Ca^{2+}-affinity aequorin probes, we have measured $[Ca^{2+}]_M$ values more than two orders of magnitude higher. We confirm here these values by making a direct in situ calibration of mitochondrial aequorin, and we show that a prolonged increase in $[Ca^{2+}]_M$ to levels of 0.5–1 mM was actually observed at any phosphate concentration (0–10 mM) during continuous perfusion of 3.5–100 μM Ca^{2+}-buffers. In spite of this high and maintained (>10 min) $[Ca^{2+}]_M$, mitochondria retained functionality and the $[Ca^{2+}]_M$ drop induced by a protonophore was fully reversible. In addition, this high $[Ca^{2+}]_M$ did not induce PTP opening unless additional activators (phenyl arsine oxide, PAO) were present. PAO induced a rapid, concentration-dependent and irreversible drop in $[Ca^{2+}]_M$. In conclusion $[Ca^{2+}]_M$ levels of 0.5–1 mM can be reached and maintained for prolonged periods (>10 min) in phosphate-containing medium, and massive opening of PTP requires additional pore activators.

1. Introduction

Along the last 15 years, measurements of mitochondrial [Ca^{2+}] have been carried out using either targeted proteins, particularly aequorin, or fluorescent dyes, mainly rhod-2. Using targeted proteins such as aequorin has the advantage of a very reliable intracellular distribution, a large dynamic range and an easy calibration in terms of the total luminescence. On the negative side, the amount of luminescence emitted is very small and hardly allows making single-cell studies. In the case of fluorescent dyes, the advantages and problems are exactly the opposite. There is plenty of fluorescence to make easily single-cell studies, but targeting is poor, the dynamic range is very small and calibration is difficult. In addition, and this is the main point we wanted to address here, both methods provide completely different values for the free mitochondrial [Ca^{2+}] that can be reached during cell stimulation.

There is a general consensus that, under resting conditions, mitochondria have low [Ca^{2+}], close to the cytosolic [Ca^{2+}] levels. In spite of the large negative electrical potential of the matrix, which provides a tremendous driving force for Ca^{2+} entry, the low Ca^{2+} affinity of the mitochondrial Ca^{2+} uniporter (MCU) keeps the Ca^{2+} entry pathway closed when the cell is under resting conditions. At the same time, the mitochondrial Na^+/Ca^{2+} exchanger uses the mitochondrial electrical potential to drive Ca^{2+} extrusion in exchange by $3Na^+$ [1], thus keeping $[Ca^{2+}]_M$ low.

Regarding cell activation, it is generally believed that MCU has little activity at cytosolic [Ca^{2+}] below 0.5 μM [2,3], but its activity increases dramatically when the extramitochondrial [Ca^{2+}] rises into the micromolar range, reaching saturation above 100 μM [3,4]. Thus, when cells are activated and the cytosolic [Ca^{2+}] increases to the micromolar range, MCU becomes activated and triggers a large flux of Ca^{2+} entry into mitochondria. However, there are large discrepancies among different techniques regarding the level of free [Ca^{2+}] that can be reached in mitochondria after MCU activation. Stimulation with histamine of intact HeLa cells loaded with rhod-2 provided peak $[Ca^{2+}]_M$ of only 2–3 μM [5]. Similarly, addition of Ca^{2+} to purified mitochondrial preparations loaded with fura2-FF gave $[Ca^{2+}]_M$ values always below 5 μM [6,7]. Instead, measurements with low-Ca^{2+}-affinity aequorin provided $[Ca^{2+}]_M$ values close to 500 μM during stimulation of intact chromaffin cells and around 1 mM after perfusion of [Ca^{2+}] buffers [4]. Moreover, stimulation with histamine of HeLa cells expressing low-Ca^{2+}-affinity

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aequorin gave $[Ca^{2+}]_M$ peak values of 20–50 μM [8,9], similar to those found using low-Ca^{2+}-affinity cameleons (50–100 μM [10]) and ratiometric pericams (10–50 μM [11]). Knowing the real $[Ca^{2+}]_M$ values during cell stimulation is critical to interpret their effects on mitochondrial metabolism, on the opening of the permeability transition pore or on the global cytosolic Ca^{2+} homeostasis. We have used here targeted aequorin to investigate this problem in more detail.

2. Methods

2.1. Cell culture and targeted aequorin expression

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 i.u. ml^{-1} penicillin and 100 i.u. ml^{-1} streptomycin. The construct for mutated aequorin targeted to mitochondria has been described previously [4]. Transfections were carried out using Metafectene (Biontex, Munich, Germany).

Bovine adrenal medulla chromaffin cells were isolated as described previously [12] and plated on 12 mm glass polilysine-coated coverslips (0.25×10^6 cells per 1 ml DMEM). Cells were cultured in high-glucose (4.5 g/l) Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 50 i.u. ml^{-1} penicillin and 50 i.u. ml^{-1} streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2. Chromaffin cells were infected with adenoviruses for expression of the mitmutAEQ construct described above. Infection was carried out the day after cell isolation and Ca^{2+} measurements were performed 24–48 h after infection.

2.2. $[Ca^{2+}]_M$ measurements with aequorin

HeLa cells were plated onto 12–13 mm round coverslips and transfected with the plasmid for mitochondrially targeted mutated aequorin. For aequorin reconstitution, HeLa cells were incubated for 1–2 h at room temperature in standard medium (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4) with 1 μM of coelenterazine n. After reconstitution, cells were placed in the perfusion chamber of a purpose-built luminometer. Then, standard medium containing 0.5 mM EGTA instead of Ca^{2+} was perfused for 1 min, followed by 1 min of intracellular medium (130 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 1–10 mM potassium phosphate, 0.5 mM EGTA, 1 mM ATP, 20 μM ADP, 10 mM L-malate, 10 mM glutamate, 20 mM Hepes, pH 7) containing digitonin (100 μM for HeLa cells, 20 μM for chromaffin cells). Then, intracellular medium without digitonin was perfused for 5–10 min, followed by buffers of known $[Ca^{2+}]$ prepared in intracellular medium using HEDTA/Ca^{2+}/Mg^{2+} mixtures. In most of the experiments, temperature was set at 22 °C in order to reduce aequorin consumption and allow longer measurements at high $[Ca^{2+}]$. Calibration of the luminescence data into $[Ca^{2+}]$ was made using an algorithm as previously described [8,13]. Statistical data are given as mean ± S.E.M.

2.3. Calibration of mutated aequorin inside mitochondria at pH 7 and pH 8

HeLa cells expressing mitochondrially targeted mutated aequorin were reconstituted with 1 μM coelenterazine n in the presence of 1 μM thapsigargin (to avoid any possible interference from the ER-Ca^{2+} store in the calibration). Then, cells were placed in the luminometer, permeabilized as described above and perfused with intracellular medium containing no ATP and no metabolic substrates, 1 mM potassium phosphate, ionophores ionomycin (1 μM) and FCCP (2 μM), 5 μM oligomycin and the SERCA pump inhibitor BHQ. In this way, mitochondria become unable to accumulate Ca^{2+} and behave simply as an aequorin container, freely permeable to Ca^{2+} and H^+, and ready to perform a calibration in the desired conditions. Cells were then perfused under these conditions with the same intracellular medium containing no EGTA and different Ca^{2+} concentrations added, from 100 to 500 μM, and buffered either at pH 7 or pH 8 (with Hepes in both cases). Luminescence data were calibrated using the same algorithm mentioned above, to study the possible discrepancies between the real $[Ca^{2+}]_M$ values (the $[Ca^{2+}]$ in the perfusion medium, in equilibrium with the mitochondrial matrix) and the calibrated ones.

2.4. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was monitored using the fluorescent indicator tetramethylrhodamine ethyl ester (TMRE). HeLa cells were mounted in a cell chamber in the stage of a Zeiss Axiovert 200 microscope under continuous perfusion, permeabilized as described above and then perfused with intracellular medium containing 5 nM TMRE until a steady-state fluorescence was reached (usually about 5 min). Single cell fluorescence was excited at 540 nm using a Cairn monochromator (200 ms excitation every 2 s) and images of the fluorescence emitted between 570 and 630 nm obtained with a 40× Fluar objective were recorded by a Hamamatsu ORCA-ER camera. Single cell fluorescence records were analyzed off-line using the Metafluor program (Universal Imaging). Experiments were performed at room temperature (22 °C).

2.5. Measurement of mitochondrially targeted EGFP fluorescence

Hela cells transfected with mitochondrially targeted EGFP were mounted in a cell chamber in the stage of a Zeiss Axiovert 200 microscope under continuous perfusion. Single cell fluorescence was excited at 480 nm using a Cairn monochromator (100 ms excitation every 2 s, 10 nm bandwidth) and images of the emitted fluorescence obtained with a 40× Fluar objective were collected using a 495DCLP dichroic mirror and a E515LPV2 emission filter (both from Chroma Technology) and recorded by a Hamamatsu ORCA-ER camera. Single cell fluorescence records were analyzed using the Metafluor program (Universal Imaging). Experiments were performed at 22 °C.

2.6. Materials

Wild type coelenterazine, coelenterazine n, and TMRE were obtained from Molecular Probes, Or. U.S.A. Other reagents were from Sigma, Madrid or Merck, Darmstadt.

3. Results

3.1. In situ calibration of mitmutAEQ

We have studied first if aequorin calibration inside mitochondria somehow differs from previous calibrations performed either in vitro or using ER-targeted aequorins [13,14]. In addition, given that mitochondrial pH is known to be about 1 pH unit higher than cytosolic pH, we have studied if increasing pH from 7 to 8 produces significant changes in aequorin luminescence. Fig. 1 shows the effect of perfusion of intracellular mediums containing known $[Ca^{2+}]$ between 100 and 500 μM in permeabilized HeLa cells expressing mutated aequorin in the mitochondrial matrix. As described in Section 2, cells were depleted of Ca^{2+}, substrates

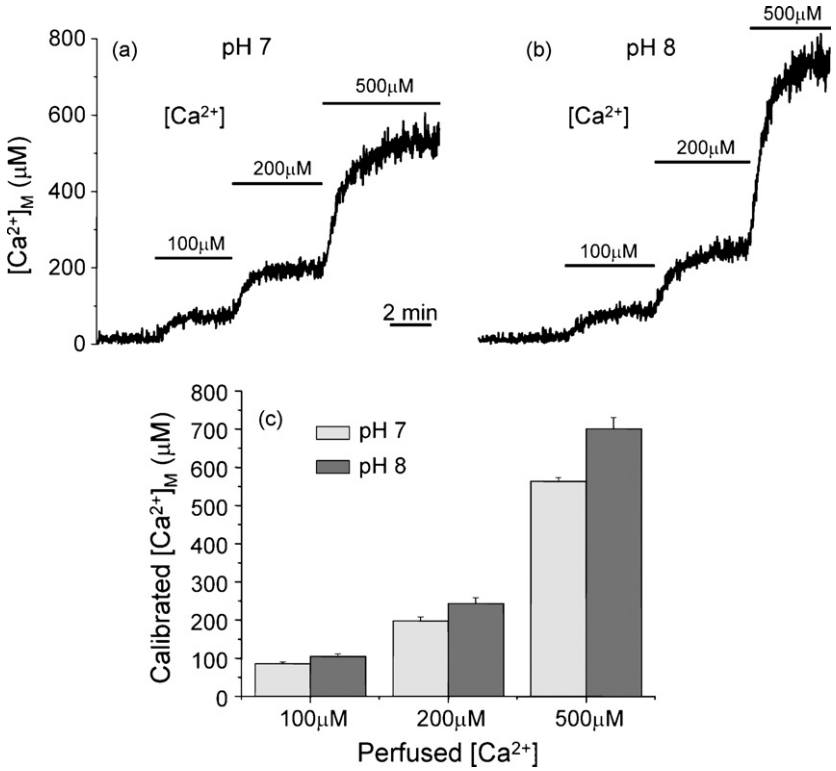

Fig. 1. In situ calibration of mitochondrially targeted mutated aequorin at pH 7 and 8. HeLa cells expressing mitmutAEQ were reconstituted with coelenterazine n in the presence of 1 μM thapsigargin. Then, cells were placed in the luminometer, permeabilized with digitonin and perfused with intracellular medium containing no ATP and no metabolic substrates, 1 μM ionomycin, 2 μM FCCP, 5 μM oligomycin and 10 μM BHQ. Cells were then perfused under these conditions with different Ca^{2+} concentrations, from 100 to 500 μM, and using intracellular medium at either pH 7 or 8 (buffered with Hepes in both cases). It can be observed that equilibrium calibrated $[Ca^{2+}]$ values are largely coincident with the corresponding $[Ca^{2+}]$ in the perfusion medium. The lower panel shows mean statistical data ± S.E. obtained with 6–8 data of each kind.

and ATP, and the mitochondrial membrane potential was abolished. Under these conditions, continuous perfusion of the Ca^{2+} solutions should lead to equilibration of the extra and intramitochondrial $[Ca^{2+}]$. The experiments were then calibrated using the same calibration obtained previously either in vitro or using ER-targeted aequorins. As can be observed in panel a (statistics in panel c), perfusion of the Ca^{2+} solutions led to an increase in calibrated $[Ca^{2+}]_M$ up to nearly the same values of $[Ca^{2+}]$ as those present in the solution perfused. Therefore, the Ca^{2+}-sensitivity of mutated aequorin inside the mitochondrial matrix is not significantly different from that previously determined in vitro or inside the ER, and the same calibration obtained before is therefore valid here. In addition, the pH increase from 7 to 8 produced only minor changes in the calibrated signal. Panel c of Fig. 1 shows that it produced a lineal increase in the calibrated $[Ca^{2+}]$ values of 22–24%. This is consistent with previous data on the pH-sensitivity of aequorin [15]. Therefore, using the calibration at pH 7 may overestimate $[Ca^{2+}]_M$ values by only this small percentage.

3.2. $[Ca^{2+}]_M$ increase in the presence of $[Ca^{2+}]$ buffers: effects of phosphate and FCCP

We have then studied the effect of perfusion of $[Ca^{2+}]$ buffers with known $[Ca^{2+}]$ on the $[Ca^{2+}]_M$ levels in permeabilized cells under more physiological conditions: in the presence of ATP and substrates, and in the absence of ionophores, so that mitochondria generate membrane potential and are able to actively accumulate Ca^{2+}. Fig. 2 shows that addition of $[Ca^{2+}]$ buffers induced an increase in $[Ca^{2+}]_M$ which was progressively faster the higher was the $[Ca^{2+}]$ in the buffer. Then $[Ca^{2+}]_M$ reached a maximum and remained there in steady-state for at least 10 min (aequorin consumption precludes longer measurements at such high $[Ca^{2+}]$). The lower panel shows statistical data on the steady-state $[Ca^{2+}]_M$ value reached at every cytosolic $[Ca^{2+}]$. Interestingly, the steady-state $[Ca^{2+}]_M$ reached was very close (700–1000 μM) for a wide range of buffered cytosolic $[Ca^{2+}]$ (5–100 μM), in spite of the large differences in the initial rate of $[Ca^{2+}]_M$ increase.

In the experiments of Fig. 2, 10 mM phosphate was included in the intracellular medium. The reason was that phosphate has been described to buffer the increase in $[Ca^{2+}]_M$ in the low micromolar range by forming a complex with Ca^{2+} [6,7]. Our data show instead that much higher $[Ca^{2+}]_M$ levels, close to millimolar, can be reached even in the presence of high phosphate. In any case, we decided to test the effect of changing the phosphate content of the intracellular medium on the $[Ca^{2+}]_M$ increase after perfusion of $[Ca^{2+}]$ buffers. Fig. 3, panel a, shows the effect on $[Ca^{2+}]_M$ of perfusion of a 3.5 μM $[Ca^{2+}]$ buffer containing 0, 1 or 3 mM phosphate at 37 °C. There were no significant changes in the rate of mitochondrial Ca^{2+} increase, in the steady state reached, or in the rate of Ca^{2+} efflux when the Ca^{2+}-buffer was substituted by intracellular medium containing EGTA. The steady-state $[Ca^{2+}]$ reached were (mean ± S.E., $n = 3$): no phosphate added, 187 ± 37 μM; phosphate 1 mM, 185 ± 20 μM; phosphate 3 mM, 193 ± 27 μM. Similarly, panel b of Fig. 3 shows the effect of perfusion of a 4.5 μM $[Ca^{2+}]$ buffer containing 0, 1, 3, 5 or 10 mM phosphate at 22 °C. Again here, no significant differences in the rate of $[Ca^{2+}]_M$ increase or in the steady-state $[Ca^{2+}]_M$ reached were observed. The steady-state $[Ca^{2+}]$ were (mean ± S.E., $n = 3$): no phosphate added, 331 ± 28 μM; phosphate 1 mM, 385 ± 23 μM, phosphate 3 mM, 327 ± 27 μM, phosphate 5 mM, 373 ± 23 μM, phosphate 10 mM, 302 ± 28 μM. Therefore, the presence of 10 mM

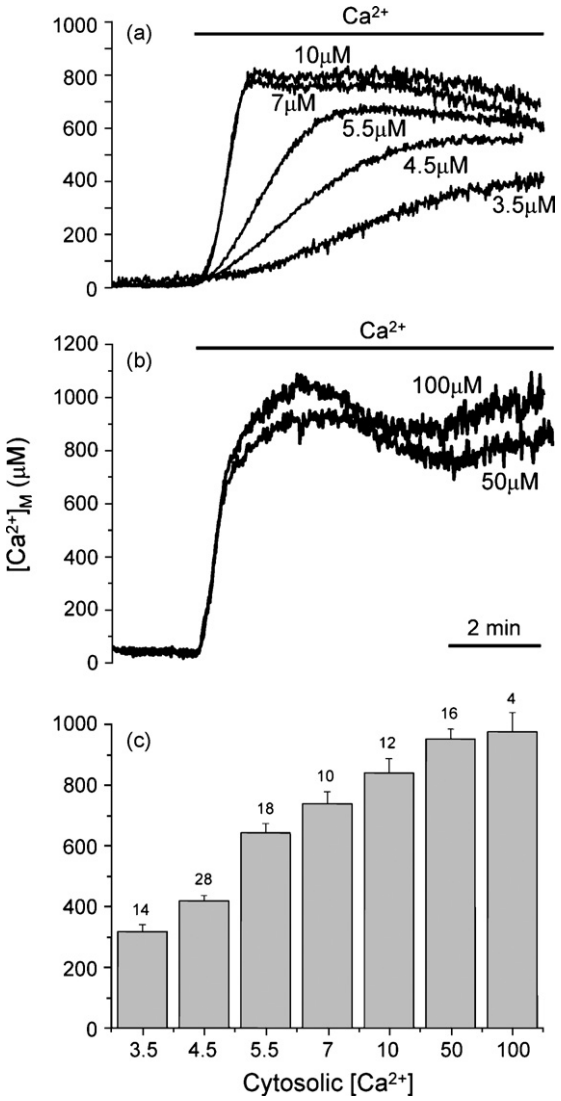

Fig. 2. Effect of continuous perfusion of [Ca^{2+}] buffers on [Ca^{2+}]$_M$. HeLa cells expressing mitmutAEQ were reconstituted with coelenterazine n, permeabilized with digitonin and perfused with intracellular medium containing 10 mM phosphate and either 3.5–10 μM [Ca^{2+}] buffers (indicated in each trace, panel a) or 50–100 μM unbuffered [Ca^{2+}] (panel b). Panel c shows statistical data of the mean ± S.E. (*n* value over each bar) steady-state [Ca^{2+}]$_M$ values reached with every [Ca^{2+}] solution.

phosphate modified little the increase in [Ca^{2+}]$_M$ up to levels of 300–400 μM. Given that precipitation of calcium phosphate ($CaHPO_4$) occurs only at [Ca^{2+}] around the millimolar range (as occurs in the endoplasmic reticulum, see Ref. [16]), we compared also the effect of the presence of high or low phosphate in the medium on larger [Ca^{2+}]$_M$ increases. Panel c of Fig. 3 shows the increase in [Ca^{2+}]$_M$ induced by a 10 μM [Ca^{2+}] buffer both in the presence of 10 mM phosphate or with no phosphate added to the medium. The figure shows that in the presence of 10 mM phosphate, the [Ca^{2+}]$_M$ increase stopped at about 800 μM, as shown in Fig. 2. However, in the absence of phosphate [Ca^{2+}]$_M$ continued increasing well above 1 mM. Even more important, if a Ca^{2+}-free buffer is perfused at this point, Ca^{2+} release was slower in the presence of high phosphate, suggesting the presence of a larger amount of total calcium in mitochondria in that condition. These data confirm that Ca^{2+} precipitation with phosphate in mitochondria occurs only or mainly when it reaches the near-millimolar range.

We have next tried to obtain information on the physiological functioning of mitochondria in the presence of high intramitochondrial [Ca^{2+}]. In Figs. 2 and 3 we showed that perfusion of Ca^{2+} buffers in permeabilized cells induces an increase in [Ca^{2+}]$_M$ that reaches an steady-state close to the millimolar range. Fig. 4 shows the effect of a 2 min treatment with the protonophore FCCP, added when [Ca^{2+}]$_M$ was at steady-state. The protonophore abolishes the mitochondrial membrane potential and the figure shows that this induced a rapid drop in [Ca^{2+}]$_M$. Then, when FCCP was washed out, mitochondria were able to restore its membrane potential, [Ca^{2+}]$_M$ increased again and finally recovered the previous steady-state. Therefore, the high steady-state [Ca^{2+}]$_M$ is maintained by the mitochondrial membrane potential, and the prolonged high [Ca^{2+}]$_M$ did not preclude mitochondria from first restoring its membrane potential after uncoupling and then take up Ca^{2+} again up to the same levels.

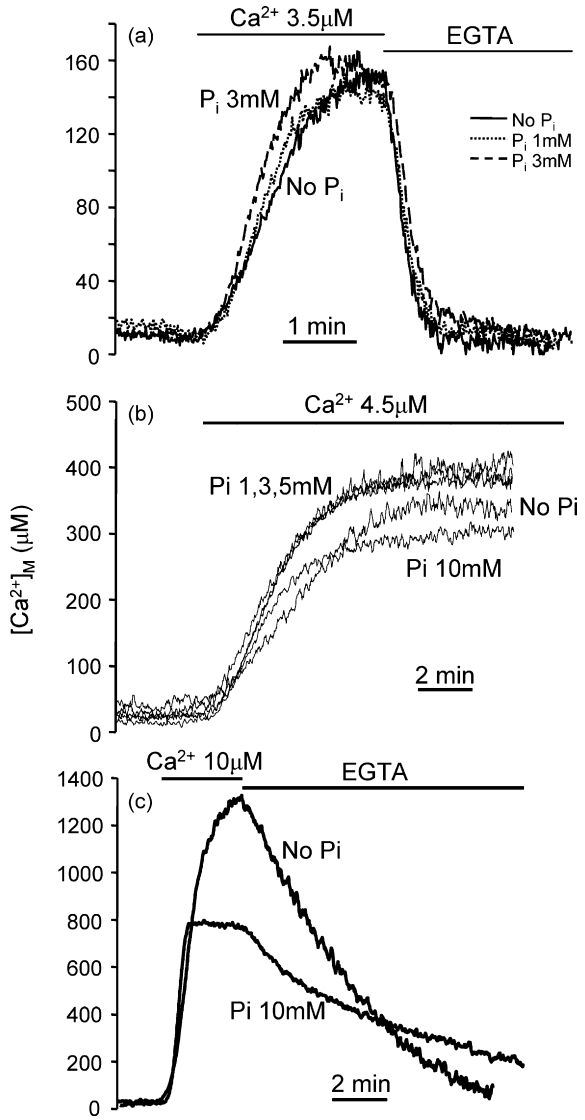

Fig. 3. Effect of phosphate concentration on the free [Ca^{2+}]$_M$ obtained after [Ca^{2+}] buffer perfusion. HeLa cells expressing mitmutAEQ were reconstituted with coelenterazine n and permeabilized in the presence of variable phosphate concentrations between 0 and 10 mM. Then, 5 min later [Ca^{2+}] buffers at the concentrations indicated in the figure, or 0.5 mM EGTA when indicated, were perfused in the same medium. Temperature: panel a, 37 °C; panels b and c, 22 °C. The experiments shown are representative of 3 similar experiments of each kind.

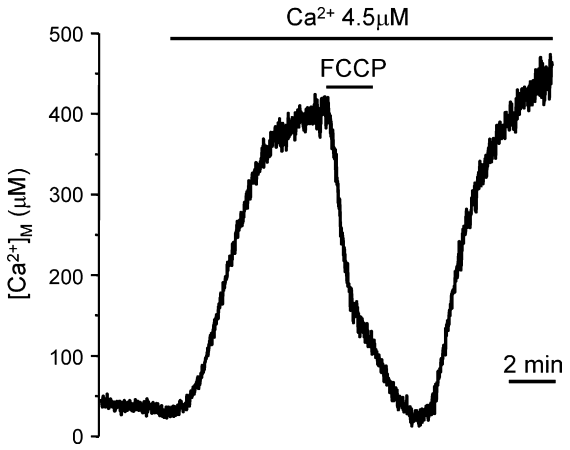

Fig. 4. Effect of FCCP on the steady-state $[Ca^{2+}]_M$. HeLa cells expressing mitmutAEQ were reconstituted with coelenterazine n, permeabilized and perfused with a 4.5 μM $[Ca^{2+}]$ buffer. Then, 2 μM FCCP was perfused for 2 min in the same buffer, as indicated, inducing a rapid and fully reversible drop in $[Ca^{2+}]_M$. The experiment shown is representative of 11 similar experiments.

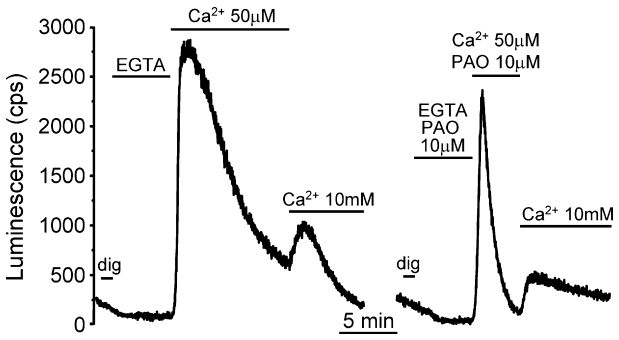

Fig. 6. Effect of treatment with PAO in the absence of Ca^{2+}. HeLa cells expressing mitmutAEQ were reconstituted with coelenterazine n and permeabilized with digitonin as indicated in the figure (dig). Then, cells were perfused for 5 min with intracellular medium containing 0.5 mM EGTA and with or without 10 μM PAO, prior to the addition of a 50 μM $[Ca^{2+}]$ solution. At the end of the experiment, a solution containing 10 mM Ca^{2+} was perfused to release the residual aequorin luminescence. The experiments shown are representative of 6–16 similar experiments of each kind.

3.3. Opening of PTP in the presence of phenyl arsine oxide (PAO): effects in $[Ca^{2+}]_M$ and mitochondrial membrane potential

Perhaps the more surprising point of the results shown above is the lack of opening of the permeability transition pore (PTP) in spite of the prolonged increase of $[Ca^{2+}]_M$ to the near-millimolar range. Opening of PTP in the large-conductance mode should produce first a rapid drop in $[Ca^{2+}]_M$ and then swelling and rupture of mitochondrial membranes. Fig. 5 shows that we can in fact trigger the opening of PTP using the widely used PTP-opener phenyl arsine oxide (PAO). This oxidant is known to induce the permeability transition thanks to its dithiol cross-linking ability [17,18]. PAO produced a rapid and concentration-dependent decrease in aequorin luminescence. In addition, particularly at high concentrations, it reduced the total number of aequorin molecules able to release photons in the preparation, as the final perfusion of 10 mM Ca^{2+} hardly produced any further release of luminescence. This phenomenon is probably due to the release of aequorin from the mitochondrial matrix as a consequence of the opening of PTP. The inset in Fig. 5 shows that the fluorescence of mitochondrially targeted EGFP, a protein with a size similar to aequorin, also starts to decrease after perfusion of PAO in the presence of Ca^{2+}, reflecting release of EGFP to the medium. Release of matrix proteins following PTP activation has been described before [19,20]. This loss of aequorin molecules completely distorts calibration and, by this reason, Fig. 5 shows only luminescence data and not calibrated $[Ca^{2+}]$. Another important question here was if the activation of PTP by PAO required a previous increase in mitochondrial $[Ca^{2+}]$. That was the case. Fig. 6 compares the luminescence records obtained after addition of Ca^{2+} to control cells or to cells treated for 5 min with 10 μM PAO in Ca^{2+}-free medium. Under control conditions, addition of Ca^{2+} triggered a fast peak of luminescence followed by a slower decrease due to aequorin consumption. In cells treated for 5 min with 10 μM PAO, the initial rate of increase in luminescence

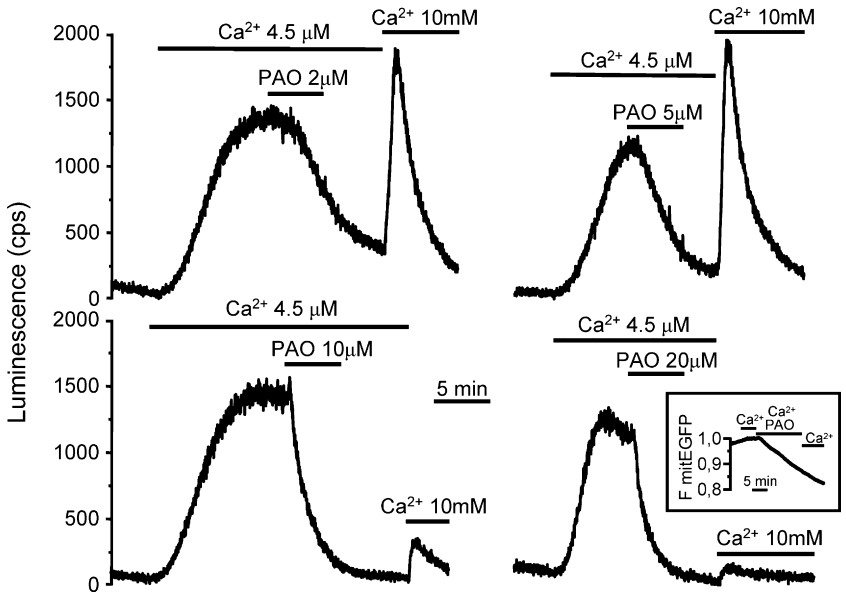

Fig. 5. Effect of phenyl arsine oxide (PAO) on the steady-state $[Ca^{2+}]_M$. HeLa cells expressing mitmutAEQ were reconstituted with coelenterazine n, permeabilized and perfused with a 4.5 μM $[Ca^{2+}]$ buffer, as shown in the figure. Then, perfusion of different concentrations of PAO, as indicated, produced a concentration-dependent decrease in luminescence. At the end of the experiment, a solution containing 10 mM Ca^{2+} was perfused to release the residual aequorin luminescence. The experiments shown are representative of 4–14 similar experiments of each kind. The inset shows the changes in normalized fluorescence of permeabilized HeLa cells expressing mitochondrially targeted EGFP after perfusion of medium containing either 10 μM Ca^{2+} or 10 μM Ca^{2+} + 20 μM PAO, as indicated. The trace shown is the average of 23 single cell traces from the same microscope field, and is representative of 4 similar experiments.

was similar, but then it was followed by a fast drop to resting levels. This indicates that 5 min treatment with PAO in the absence of Ca^{2+} was unable to open PTP, so mitochondria were still able to take up Ca^{2+}. Then, the $[Ca^{2+}]_M$ increase in the presence of PAO rapidly activated PTP, inducing a sudden release of the accumulated Ca^{2+}. In conclusion, both PAO and an increase in $[Ca^{2+}]_M$ were required to trigger PTP opening.

Further evidence for the effect of PAO on PTP opening was obtained by measuring their effects on mitochondrial membrane potential, estimated with the dye TMRE. Fig. 7, panel a, shows that perfusion of a Ca^{2+} buffer alone induced a small mitochondrial depolarization. Then, still in the continuous presence of the Ca^{2+} buffer, mitochondrial membrane potential returned to the original values 20–30 min afterwards. The inset shows that the magnitude of the depolarization was proportional to the cytosolic $[Ca^{2+}]$ in the buffer, and the kinetics was similar to that of the $[Ca^{2+}]$ changes (see Fig. 2). Panels b and c show that addition of PAO, either a short pulse of a high concentration (10 μM) or the continuous perfusion of a low concentration (1 μM), strongly accelerated mitochondrial depolarization, and mitochondria were then unable to recover the original potential.

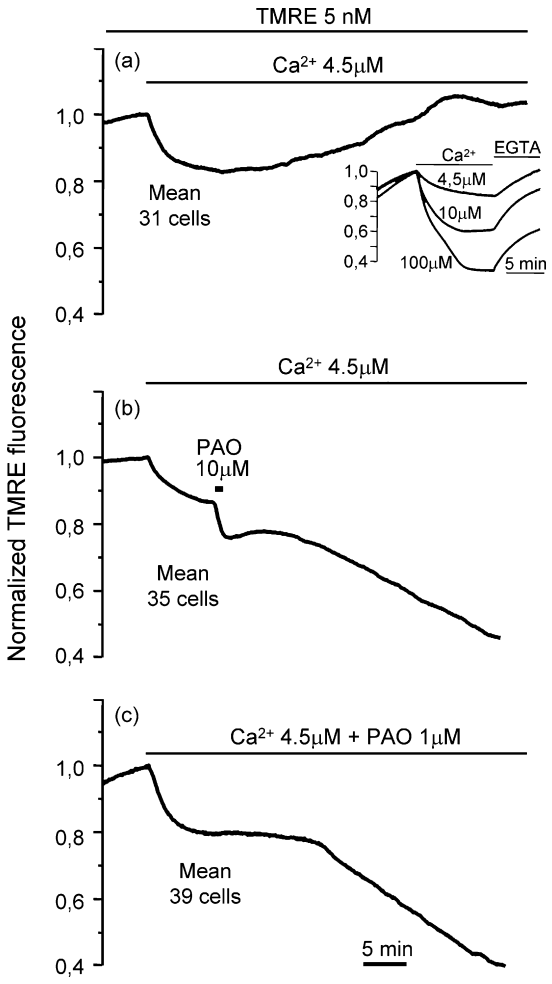

Fig. 7. Effects of Ca^{2+} and PAO on mitochondrial membrane potential. HeLa cells were mounted in a cell chamber in the stage of a Zeiss Axiovert 200 microscope under continuous perfusion, permeabilized with digitonin and then perfused with intracellular medium containing 5 nM TMRE until a steady-state fluorescence was reached (usually about 5 min). Then, a 4.5 μM $[Ca^{2+}]$ buffer with or without PAO was added as indicated. The traces shown are the mean of those obtained in all the cells present in the microscope field in each case, as indicated. The experiments shown are representative of 13–24 similar experiments of each kind. The inset in panel a shows the effect on mitochondrial membrane potential of perfusion of Ca^{2+} buffers containing 4.5 μM (average of 34 single-cell records), 10 μM (average of 33 single-cell records) or 100 μM (average of 38 single-cell records) $[Ca^{2+}]$. Data in the inset are representative of 3–4 separate experiments of each kind.

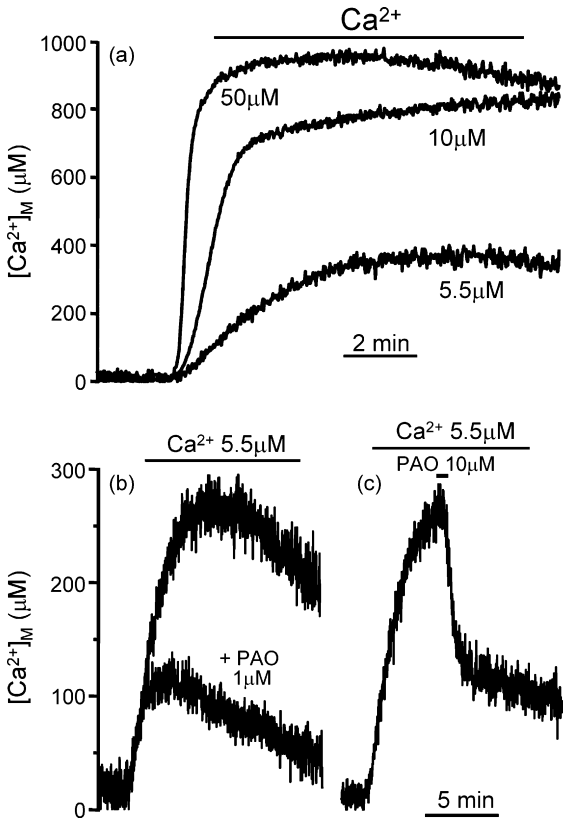

Fig. 8. Effects of Ca^{2+} buffers and PAO on $[Ca^{2+}]_M$ in chromaffin cells. Chromaffin cells expressing mitmutAEQ were reconstituted with coelenterazine n, permeabilized with digitonin and perfused with a 5.5 μM Ca^{2+} buffer. When indicated, either 1 μM PAO was present all the time together with the Ca^{2+} buffer (panel b), or 10 μM PAO was added for only 1 min (panel c). The experiments shown are representative of 3–11 similar experiments of each kind.

3.4. $[Ca^{2+}]_M$ in primary chromaffin cells: effects of $[Ca^{2+}]$ buffers and PAO

We have finally investigated if a primary cell culture behaves in the same way as the immortal HeLa cell cultures. Fig. 8 shows data obtained in primary cultures of bovine adrenal medulla chromaffin cells. Panel a shows that addition of Ca^{2+} buffers to permeabilized chromaffin cells produced an increase in $[Ca^{2+}]_M$ that reached an steady-state close to 1 mM. In the lower panels, the effect of perfusion of PAO is shown. Panel b shows the effect of continuous perfusion of 1 μM PAO and panel c shows the effect of a short pulse of 10 μM PAO. In both cases, this oxidant induced a drop in $[Ca^{2+}]_M$, as occurred in HeLa cells. In chromaffin cells, however, we did not detect a loss of aequorin after addition of PAO, so that the addition of high Ca^{2+} at the end of the experiment triggered a peak of luminescence similar to those obtained in the controls. We have therefore shown the calibrated values in Fig. 8.

4. Discussion

In this paper we have investigated the behaviour of mitochondrial $[Ca^{2+}]$ in the presence of a prolonged (>10 min) increase in cytosolic $[Ca^{2+}]$ in the micromolar range. We show first that the Ca^{2+}-sensitivity of mutated aequorin reconstituted with coelenterazine n inside mitochondria is the same as that previously determined in vitro or inside the ER [13,14]. Thus, all the cali-

brated $[Ca^{2+}]_M$ values obtained in this and previous papers with that aequorin form targeted to mitochondria are clearly valid. We have to remark this point because an in situ calibration of native aequorin reconstituted with native coelenterazine [21] showed important differences with our previously made in vitro calibration of that type of aequorin in the submicromolar $[Ca^{2+}]$ range. This argument, together with the problem of aequorin consumption, has been later used to throw doubts on the validity of all the previous work made with low-Ca^{2+}-affinity mitochondrial aequorin [6]. Our data here demonstrate that the low-Ca^{2+}-affinity mutated aequorin is perfectly suitable to measure $[Ca^{2+}]$ inside mitochondria and our previous calibration of mutated aequorin reconstituted with coelenterazine n [13] can confidently be used when this chimera is targeted to the mitochondrial matrix. Moreover, aequorin consumption is not a problem to monitor high $[Ca^{2+}]_M$ for at least 10 min, as we have shown before using the same aequorin form targeted to the endoplasmic reticulum [13]. In any case, we should also mention that the comparison made by Pitter et al. [21], between their in situ calibration and our previous in vitro calibration [22] was made under different conditions of $[Mg^{2+}]$ and temperature, two factors that considerably modify the Ca^{2+}-sensitivity of aequorin [15,23], and using a different combination of chelators to prepare the submicromolar Ca^{2+} buffers. Thus, in our opinion, confirmation of the different Ca^{2+}-sensitivity in situ and in vitro of native aequorin still awaits making the comparison under the same conditions.

We find here that increasing the cytosolic $[Ca^{2+}]$ to the micromolar range leads to prolonged increases in $[Ca^{2+}]_M$ with steady-state values close to 1 mM. Increasing the phosphate content of the medium had an effect on $[Ca^{2+}]_M$ increase or Ca^{2+} release from mitochondria only when it reached the near-millimolar range, as expected from the solubility of the $CaHPO_4$ complex in ionic solutions or in other cellular compartments such as the endoplasmic reticulum (16). Our data strongly contrast with those obtained with fluorescent dyes such as fura2-FF in purified mitochondrial preparations [6,7], which showed much lower maximum free $[Ca^{2+}]$ values (below 5 μM) in the mitochondrial matrix. We should mention here that it is now widely accepted that the ER has a $[Ca^{2+}]$ in the 300–500 μM range. However, at the time we first measured these values (using the same mutated aequorin reconstituted with coelenterazine n used here, but targeted to the ER [14]), data obtained with low-Ca^{2+}-affinity fluorescent dyes were also giving much lower values for $[Ca^{2+}]_{ER}$ [24–27]. Later evidence, obtained either with targeted fluorescent cameleons [10,28] or with fluorescent dyes but correcting for mislocalization and other problems [29,30], changed the views in favour of $[Ca^{2+}]_{ER}$ values closer to those obtained with low-Ca^{2+}-affinity aequorin. Regarding mitochondria, data obtained with low-Ca^{2+}-affinity cameleons [10] and ratiometric pericams [11] targeted to mitochondria have also confirmed the $[Ca^{2+}]$ values obtained with low-Ca^{2+}-affinity aequorin for the histamine-induced mitochondrial $[Ca^{2+}]$ peak in HeLa cells.

It has been reported that the mitochondrial Ca^{2+} uniporter is inactivated by Ca^{2+} in the high micromolar range [31]. We could not detect that effect during continuous perfusion of a Ca^{2+} buffer. Similarly, the rate of $[Ca^{2+}]_M$ increase was not modified by previous (10–15 min) perfusion of a 1 min pulse with a 10 μM Ca^{2+} buffer (data not shown). On the other hand, it was surprising to find that the prolonged (>10 min) increases in $[Ca^{2+}]_M$ in the millimolar range were unable to induce by themselves opening of PTP, both in HeLa cells and in primary cultures of chromaffin cells. In addition, mitochondrial functionality was apparently well preserved. The mitochondrial membrane potential was maintained during Ca^{2+}-buffer perfusion (Fig. 7a) and depolarization with FCCP induced a rapid but fully reversible release of Ca^{2+} from mitochondria, showing that mitochondria are able to restore its membrane potential and take up Ca^{2+} again up to the same levels (Fig. 4). Opening of PTP was rapidly triggered, however, when we added the classical PTP inducer phenyl arsine oxide in the presence of Ca^{2+} (but not in the absence). Our data thus confirm the classical idea that opening of PTP depends of many different factors, including Ca^{2+}, pH, membrane potential, thiol oxidation, etc., and that it behaves as a sort of coincidence detector, that requires more than one factor present to activate [32,33]. It is remarkable, in any case, the finding that mitochondria are able to maintain such a high $[Ca^{2+}]_M$ for many minutes with no apparent loss of functionality.

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