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Dynamics of mitochondrial [Ca^{2+}] measured with the low-Ca^{2+}-affinity dye rhod-5N

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ABSTRACT

Available methods to measure mitochondrial [Ca^{2+}] ($[Ca^{2+}]_M$) include both targeted proteins and fluorescent dyes. Targeted proteins usually report much higher $[Ca^{2+}]_M$ values than fluorescent dyes, up to two orders of magnitude. However, we show here that the low-Ca^{2+}-affinity dye rhod-5N provides $[Ca^{2+}]_M$ values similar to those reported by targeted aequorin, suggesting that the discrepancies are mainly due to the higher Ca^{2+}-affinity of the fluorescent dyes used. We find rhod-5N has an apparent *in situ* intramitochondrial Kd around 0.5 mM. Addition of Ca^{2+} buffers containing between 4.5 and 10 μM [Ca^{2+}] to permeabilized cells loaded with rhod-5N induced increases in calibrated $[Ca^{2+}]_M$ up to the 100 μM–1 mM range, which were dependent on mitochondrial membrane potential. Ca^{2+} release from mitochondria was largely dependent on [Na^+]. We have then used rhod-5N loaded cells to investigate the $[Ca^{2+}]_M$ response to agonist stimulation at the single-cell and subcellular level.The $[Ca^{2+}]_M$ peaks induced by histamine varied by nearly 10-fold among different cells, with a mean about 25 μM. In the presence of the Ca^{2+} uniporter stimulator kaempferol, the $[Ca^{2+}]_M$ peaks induced by histamine were also highly variable, and the mean $[Ca^{2+}]_M$ peak was 3-fold higher. Simultaneous measurement of cytosolic and mitochondrial [Ca^{2+}] peaks showed little correlation among the heights of the peaks in both compartments. Studying the $[Ca^{2+}]_M$ peaks at the subcellular level, we found significant heterogeneities among regions in the same cell. In particular, the $[Ca^{2+}]_M$ increase in mitochondrial regions close to the nucleus was more than double that of mitochondrial regions far from the nucleus.

1. Introduction

Evidence for the multiple roles played by mitochondria in cell Ca^{2+} homeostasis has been continuously growing in the last years. Activation of mitochondrial metabolism, induction of apoptosis, modulation of cytosolic [Ca^{2+}] through transient Ca^{2+} buffering of cytosolic Ca^{2+} microdomains, modulation of ER-Ca^{2+} release or modulation of Ca^{2+} entry through the plasma membrane, are just some of the roles which have been attributed to the mitochondria in Ca^{2+} homeostasis [1,2]. However, there are still significant discrepancies in the $[Ca^{2+}]_M$ measurements obtained by different procedures, and the low-Ca^{2+}-affinity forms of targeted proteins such as aequorin or camaleon [3–5] report much higher $[Ca^{2+}]_M$ values than fluorescent dyes such as rhod-2 or fura-FF [6,7]. We have studied before this discrepancy and we have shown that the *in situ* intramitochondrial calibration of aequorin does not significantly differ from that obtained in vitro or in a different intracellular location [8], suggesting that measurements with this photoprotein are correct. We have also recently reported that rhod-2 is unable to follow adequately repetitive changes in $[Ca^{2+}]_M$, while measurements obtained with aequorin and the fluorescent protein pericam are consistent [9]. In addition, kinetic studies of mitochondrial Ca^{2+} fluxes indicate that massive calcium phosphate complexes precipitation inside mitochondria only occurs when $[Ca^{2+}]_M$ reaches the millimolar range [10].

However, in spite of these problems, fluorescent dyes such as rhod-2 still have several important advantages over aequorin which favour a much wider use, namely the easy loading even in cells difficult to transfect and also the ability to make single cell or subcellular studies. Thus, we have decided to test if the discrepancies could be solved at least in part by using a fluorescent dye with a much lower Ca^{2+} affinity. In this paper we have used rhod-5N, a very low-Ca^{2+}-affinity derivative of rhod-2 (in vitro Kd for Ca^{2+} binding of 320 μM), to study Ca^{2+} dynamics in HeLa cells and make a comparison with the previous data obtained with aequorin. Our data demonstrate that the $[Ca^{2+}]_M$ data obtained with rhod-5N are fully consistent, both in terms of kinetics and of absolute $[Ca^{2+}]_M$ values, with those obtained previously with aequorin. Moreover, this dye allows obtaining important new data about $[Ca^{2+}]_M$ dynamics at the single cell and subcellular level.

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2. Methods

2.1. Cell culture and perfusion media

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 100 i.u. ml^{-1} penicillin and 100 i.u. ml^{-1} streptomycin. Cells were then plated onto 12 mm round coverslips and loaded with rhod-5N by incubation in standard medium containing 2 μM of the acetoxymethyl ester derivative (rhod-5N-AM, Anaspec, CA, USA) during 30 min at room temperature. Cells were then washed with standard medium for 30 min at room temperature and mounted in the cell chamber in the stage of a Zeiss Axiovert 200 microscope under continuous perfusion. Simultaneous loading with rhod-5N and fura-2 was made by incubating cells in standard medium containing 2 μM of both rhod-5N-AM and fura-2-AM during 30 min at room temperature, followed by 30 min wash. Standard extracellular medium contained 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4. For experiments with permeabilized cells, 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 mM potassium phosphate, 0.5 mM EGTA, 1 mM ATP, 20 μM ADP, 5 mM L-malate, 5 mM glutamate, 5 mM succinate, 20 mM Hepes, pH 7) containing 100 μM digitonin. Then, intracellular medium without digitonin was perfused for 5 min prior to the experiment. *In situ* calibration of rhod-5N was made in HeLa cells loaded with rhod-5N and permeabilized as above. After permeabilization, cells were perfused with medium containing 130 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 4 μM FCCP, 20 mM Hepes, pH 8, and different $[Ca^{2+}]$ between 50 μM and 10 mM (see Fig. 1).

2.2. $[Ca^{2+}]_M$ measurements with rhod-5N

Single cell fluorescence was excited at 550 nm using a Cairn monochromator (300 ms excitation every 2 s, 10 nm bandwidth) and images of the emitted fluorescence obtained with a 63× objective were collected using a 565DCLP dichroic mirror and a E590LPV2 emission filter (both from Chroma Technology) and recorded by a Hamamatsu ORCA-ER camera. Experiments were performed at room temperature (22 °C). $[Ca^{2+}]_M$ was calculated from the calibration as described in Section 3. Simultaneous monitoring of rhod-5N and fura-2 fluorescence was achieved by consecutive excitation at 340, 380 and 550 nm using a Cairn monochromator and a 73100 beamsplitter from Chroma Technology. In these experiments, the fluorescence images were collected with a 40× Fluar objective using a 73101 emission filter from Chroma Technology. To calculate cytosolic $[Ca^{2+}]$, single cell fluorescence records obtained at 340 and 380 nm excitation were ratioed and calibrated into $[Ca^{2+}]$ values off-line as described before [11], using the Metafluor program (Universal Imaging).

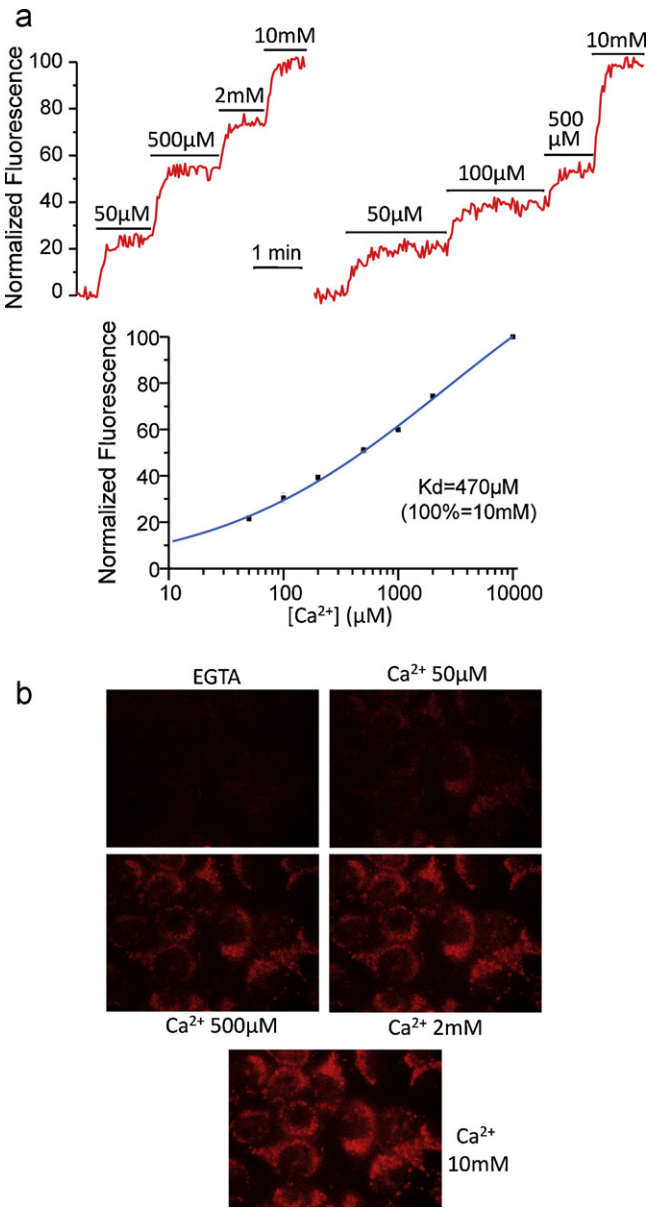

Fig. 1. Intracellular calibration of rhod-5N fluorescence. HeLa cells were loaded with rhod-5N, permeabilized and then perfused with solutions containing 130 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 4 μM FCCP, 20 mM Hepes, pH 8, and different $[Ca^{2+}]$ between 50 μM and 10 mM, as indicated. (a) Typical normalized records obtained by taking the fluorescence obtained at 10 mM $[Ca^{2+}]$ as 100% and the resting fluorescence obtained in EGTA-containing medium as 0%. The mean values obtained at every $[Ca^{2+}]$ (from 3 to 6 data at each point) were plotted in the lower panel and the sigmoidal fitting of the data was used to calibrate the rest of the experiments throughout this paper. (b) Images from a typical experiment showing the progressive increase in fluorescence obtained after perfusion of the calibration solutions.

3. Results

Rhod-5N is a very low-Ca^{2+}-affinity rhod-2 derivative (in vitro Kd = 320 μM). To estimate the real Kd under physiological conditions inside mitochondria, we have first made an *in situ* intracellular calibration. Cells loaded with the dye were permeabilized and perfused with solutions containing known $[Ca^{2+}]$ and FCCP, as described in Section 2, to depolarize mitochondria and equilibrate the $[Ca^{2+}]$ in the medium and in the organelle. The upper panel of Fig. 1a shows that addition of each $[Ca^{2+}]$ induced a step-like increase in rhod-5N fluorescence, suggesting that under these conditions there is a fast equilibration of the $[Ca^{2+}]$ between the medium and the mitochondrial matrix space. Fig. 1b shows a series of images corresponding to a typical calibration experiment, to show the changes in fluorescence following perfusion of the calibration solutions. The lower panel of Fig. 1a shows the calibration curve obtained from a series of experiments like those of the upper panel, and the sigmoidal fitting of the data, as corresponds for a typical ligand-binding process. Assuming the fluorescence value obtained at 10 mM $[Ca^{2+}]$ as 100%, 50% fluorescence was obtained at 470 μM $[Ca^{2+}]$. In fact, the rhod-5N fluorescence would probably increase still further at $[Ca^{2+}]$ above 10 mM, so that the real Kd would be higher, but that range was not considered here. This calibration curve, obtained after sigmoidal fitting of the data, was used throughout the paper to transform fluorescence values into $[Ca^{2+}]$,

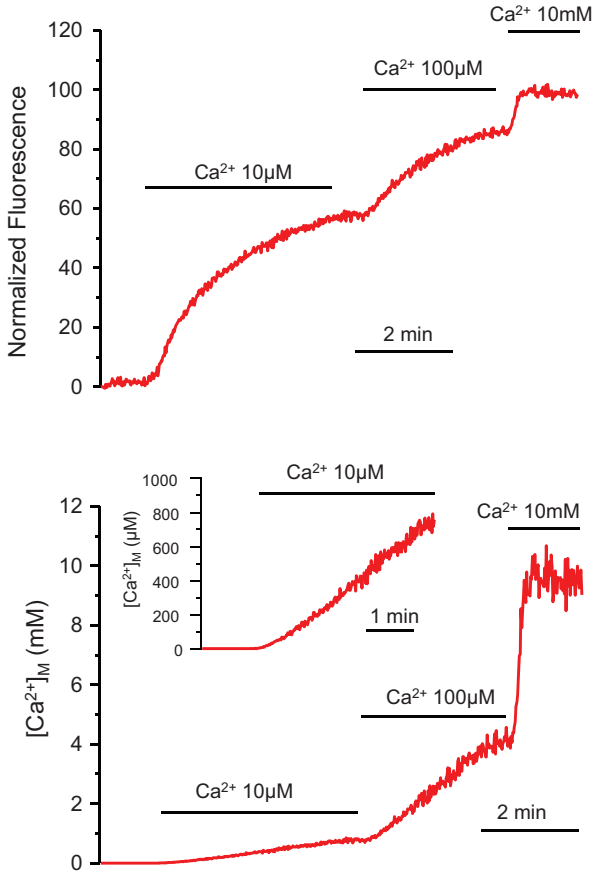

Fig. 2. Calibration of mitochondrial Ca^{2+} uptake in permeabilized cells. HeLa cells were loaded with rhod-5N, permeabilized and then perfused with solutions containing intracellular medium and 10 μM or 100 μM [Ca^{2+}], as indicated. At the end of every experiment, a calibration solution containing 10 mM [Ca^{2+}] was always added. The upper panel shows a normalized typical fluorescence record and the lower panel shows the same experiment calibrated into [Ca^{2+}] values (the inset shows at an expanded scale the effect of the 10 μM [Ca^{2+}] buffer).

after normalization of the fluorescence records using the fluorescence obtained with the 10 mM [Ca^{2+}] medium as 100% and the resting fluorescence as 0%.

We have then studied the effect of perfusing controlled [Ca^{2+}] in permeabilized HeLa cells on $[Ca^{2+}]_M$ measured with rhod-5N. Fig. 2 shows that perfusion of a [Ca^{2+}] buffer containing 10 μM [Ca^{2+}] induced an increase in rhod-5N fluorescence that reached around 60% of the maximum fluorescence within 5 min and stabilized there. Subsequent addition of medium containing 100 μM [Ca^{2+}] induced a further increase in $[Ca^{2+}]_M$ reaching nearly 90% of the maximum fluorescence. In the lower panel, the fluorescence data of the upper panel have been calibrated into [Ca^{2+}] values using the calibration curve of Fig. 1. The inset shows the effect of the perfusion of the 10 μM [Ca^{2+}] buffer at an expanded scale, showing that $[Ca^{2+}]_M$ increases up to values close to 1 mM, a value consistent with those obtained with low-Ca^{2+}-affinity aequorin under similar conditions [8–10]. Then, the addition of 100 μM [Ca^{2+}] to the extramitochondrial space still induced a further increase in $[Ca^{2+}]_M$ up to about 4 mM.

Fig. 3a shows that the rate of increase in $[Ca^{2+}]_M$ was dependent on the [Ca^{2+}] in the perfusion medium. The calibrated values (right panel) show the large difference in $[Ca^{2+}]_M$ accumulation obtained at 4 5, 7 and 10 μM [Ca^{2+}] in the perfusion medium. In panel (B), the rate of Ca^{2+} release from mitochondria was measured by loading first mitochondria with Ca^{2+} and then shifting the perfusion medium to an EGTA-containing Ca^{2+} free medium. Fig. 3b shows that the rate of Ca^{2+} release from mitochondria was highly dependent on the [Na^+] of the medium. Again this kind of Ca^{2+}-release curves were very similar to those obtained with low-Ca^{2+}-affinity aequorin [10] and show the importance of the Na^+/Ca^{2+} exchanger for Ca^{2+} release from mitochondria in HeLa cells. The residual Ca^{2+} release rate observed in Na^+-free medium may be attributed to Na^+-independent mitochondrial Ca^{2+}-release pathways such as the H^+/Ca^{2+} exchange. Fig. 3c shows that the Ca^{2+} accumulation in mitochondria measured by rhod-5N was also strictly dependent on the presence of a membrane potential in the mitochondria. Addition of the protonophore FCCP after Ca^{2+}-accumulation immediately induced a fast Ca^{2+} release from mitochondria, as previously reported using aequorin [8].

The data above show that rhod-5N is able to measure $[Ca^{2+}]_M$ and provides $[Ca^{2+}]_M$ values and dynamic measurements close to those reported by low-Ca^{2+}-affinity aequorin. We have then used this dye to investigate agonist-induced $[Ca^{2+}]_M$ signaling at the single cell level. Fig. 4 shows the $[Ca^{2+}]_M$ transients induced by histamine in single HeLa cells. In order to calibrate the experiments in [Ca^{2+}], cells were first stimulated with histamine and then permeabilized and perfused with the 10 mM Ca^{2+}-containing calibration solution. Experiments were then normalized using that fluorescence value as 100% and the resting fluorescence as 0%, and the calibration curve of Fig. 1 was then applied to obtain absolute values of $[Ca^{2+}]_M$. Fig. 4a shows four single-cell normalized fluorescence traces and the translation into $[Ca^{2+}]_M$ values using the calibration curve, to show the procedure. Fig. 4b, panels A, B, and C, shows the histamine-induced $[Ca^{2+}]_M$ peaks obtained in 11 different cells present in the microscope field. Panel D shows the mean $[Ca^{2+}]_M$ increase of all those cells, and panel E shows the mean of three similar experiments. We can see that there is a significant variability among the $[Ca^{2+}]_M$ peak in different cells, ranging between less that 10 μM up to more than 60 μM. The mean value was around 25 μM, and this value is consistent with our previous data obtained with low-Ca^{2+}-affinity aequorin [12,13]. We have then studied the effect of the stimulation of the mitochondrial Ca^{2+} uniporter with the flavonoid kaempferol [13]. Fig. 5 shows that this flavonoid induced a large increase in the $[Ca^{2+}]_M$ peaks induced by histamine in single HeLa cells. As in the previous figure, panels A, B and C show single cell data, panel D shows the mean of that experiment and panel E shows the mean of three similar experiments. The single cell data show again a large variability in the $[Ca^{2+}]_M$ response among different cells, with the peak ranging between 30 μM and 400 μM. The mean was around 70 μM, a value again consistent with the previous data we have reported using low-Ca^{2+}-affinity aequorin [13].

We have reported recently that rhod-2 is not able to monitor adequately $[Ca^{2+}]_M$ when repetitive stimulation protocols are used [9]. Unfortunately, the same problem also affects rhod-5N, so that a second stimulation with histamine induced a much smaller response than the first (data not shown). Therefore, this dye should be better used only for short or single-stimulation protocols.

The spectral properties of rhod-2 allow simultaneous recording of its fluorescence and that of fura-2 excited at 340 and 380 nm to monitor cytosolic [Ca^{2+}]. We have used this possibility to compare the cytosolic and mitochondrial [Ca^{2+}] peaks at the single cell level. The upper panels of Fig. 6 show a series of single-cell [Ca^{2+}] peaks obtained in parallel in cytosol and in mitochondria, and the lower panel shows the relationship among the cytosolic and mitochondrial [Ca^{2+}] peaks obtained in 23 different cells. The data show that there is considerable dispersion and little correlation among the height of the [Ca^{2+}] peaks in both compartments. This suggests that the size of the $[Ca^{2+}]_M$ peak may not only depend on the size of the $[Ca^{2+}]_c$ peak, and additional factors could contribute to modulate mitochondrial [Ca^{2+}] uptake.

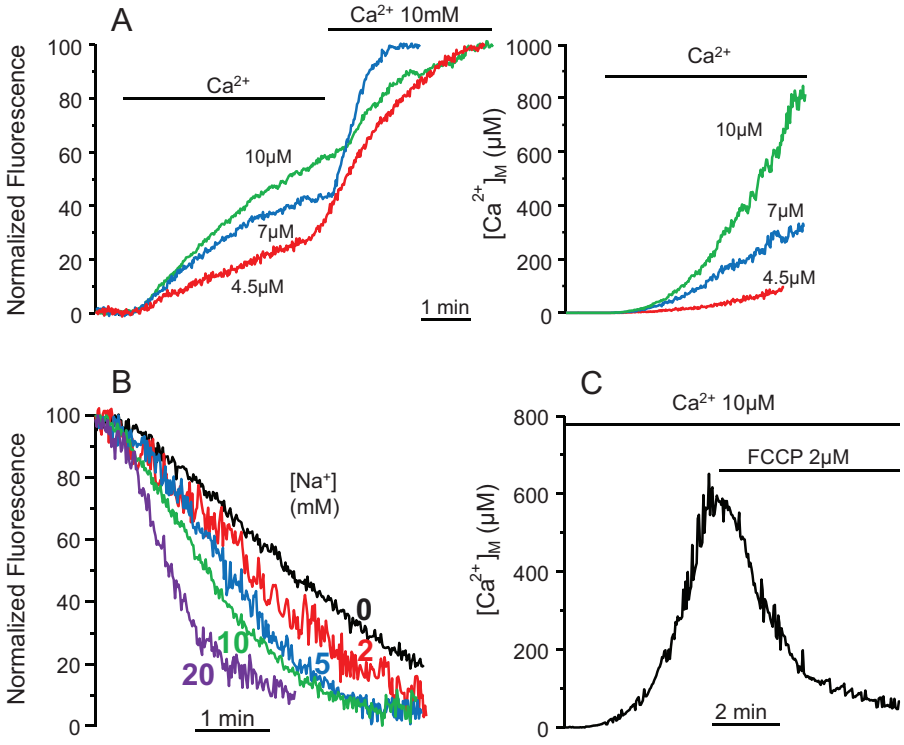

Fig. 3. Dynamics of $[Ca^{2+}]$ in mitochondria of permeabilized cells. HeLa cells were loaded with rhod-5N and permeabilized. (a) Cells were then perfused with solutions containing intracellular medium and either 4.5, 7 or 10 μM $[Ca^{2+}]$, as indicated. The curves in the left show the normalized fluorescence records and those in the right show the calibrated values. (b) Cells were perfused with a 10 μM $[Ca^{2+}]$ buffer and then shifted to an EGTA-containing medium to start Ca^{2+} release in the presence of different $[Na^+]$, as indicated. (c) Cells were perfused with a 10 μM $[Ca^{2+}]$ buffer and then 2 μM FCCP was added as indicated.

We have finally studied if rhod-5N could detect subcellular variability in the $[Ca^{2+}]_M$ peaks induced by histamine. For this study we have preferentially used cells with a more elongated shape, so that regions close and far from the nucleus could be easily distinguished. Fig. 7 shows the $[Ca^{2+}]_M$ peaks obtained in 5 subcellular regions of one of the analyzed cells. To maximize fluorescence, the image shown was obtained after perfusion of the 10 mM $[Ca^{2+}]$ calibration medium. Unfortunately, the level of rhod-5N loading was not high enough to provide good images of the $[Ca^{2+}]_M$ peak. The non-tubular aspect of mitochondria is due to cell permeabilization and the same images are obtained using cells expressing mitochondrially targeted EGFP (data not shown). It is apparent that the regions close to the nucleus (2 and 3) in this cell generate a higher $[Ca^{2+}]_M$ transient than the rest. We have thus made a more systematic analysis of regions close and far from the nucleus in 10 different cells and we have found that the mean $[Ca^{2+}]_M$ increase in regions close to the nucleus was about twice that obtained in regions far from the nucleus (2.2 ± 0.2 fold, mean ± S.E., $n = 10$).

4. Discussion

Mitochondrial Ca^{2+} dynamics at the single cell or subcellular level has been mainly studied using high-Ca^{2+}-affinity dyes such as rhod-2 [6,14–16], whose *in situ* Kd for Ca^{2+} binding is around 1 μM, and the maximum $[Ca^{2+}]_M$ values obtained after cell stimulation have been around 2–4 μM. In contrast, the fluorescent protein pericam, which has an apparent intramitochondrial Kd for Ca^{2+} of 11 μM, measured mean $[Ca^{2+}]_M$ values of 10 μM and maximum values well above 50 μM in some subcellular regions [5]. Similarly, a low-Ca^{2+}-affinity cameleon protein provided maximum values of nearly 100 μM for the $[Ca^{2+}]_M$ increase after histamine stimulation of HeLa cells [4], values close to those obtained with the luminescent protein aequorin in cell populations [12,13].

We have recently described that rhod-2 has some difficulties to monitor adequately $[Ca^{2+}]_M$ during repetitive cell stimulation. However, the discrepancy in the size of the initial $[Ca^{2+}]_M$ peak may also be due to the high affinity of the dye. In fact, the lower affinity dye rhod-FF (Kd = 19 μM) has been used in some cases to avoid saturation with Ca^{2+} or to minimize the signal coming from mistargeted cytosolic dye [17,18]. In these papers, although the data were not calibrated in $[Ca^{2+}]$ values, it was actually shown that rhod-2 saturated with Ca^{2+} in mitochondria during cell stimulation, while rhod-FF had a better response. The very low Ca^{2+}-affinity dye rhod-5N (in vitro Kd = 320 μM) we use in this paper has been previously used once to study the effect of trains of action potentials on $[Ca^{2+}]_M$ in motor nerve terminals [19]. In that work, cell stimulation induced a very significant increase in rhod-5N fluorescence that nearly doubled the resting fluorescence. However, calibration of the data by the authors led to matrix $[Ca^{2+}]$ values of only 1 μM, by using systematically an F_{max}/F_{min} quotient of 200 for rhod-5N fluorescence. In our experiments, the mean F_{max}/F_{min} quotient was 3.3 ± 0.4 (mean ± S.E., $n = 11$), and was somewhat variable depending on the signal to noise ratio. Thus, we have preferred to calibrate each experiment by measuring the fluorescence of the 10 mM $[Ca^{2+}]$ medium at the end of every experiment and then use it to apply the calibration curve of Fig. 1.

In this paper we have investigated the ability of rhod-5N to monitor $[Ca^{2+}]_M$ in intact and permeabilized HeLa cells and we have made an *in situ* calibration of the dye to be able to provide absolute $[Ca^{2+}]$ values. According to our *in situ* calibration, the apparent Kd for Ca^{2+}-binding of rhod-5N inside mitochondria was 470 μM taking the fluorescence obtained at 10 mM $[Ca^{2+}]$ as the maximum. In fact, the calibration curve suggested that reaching the maximum fluorescence required higher $[Ca^{2+}]$, so that the real Kd is probably higher, but that was not further studied because these concentrations are far from the physiological range. The *in situ* Kd obtained

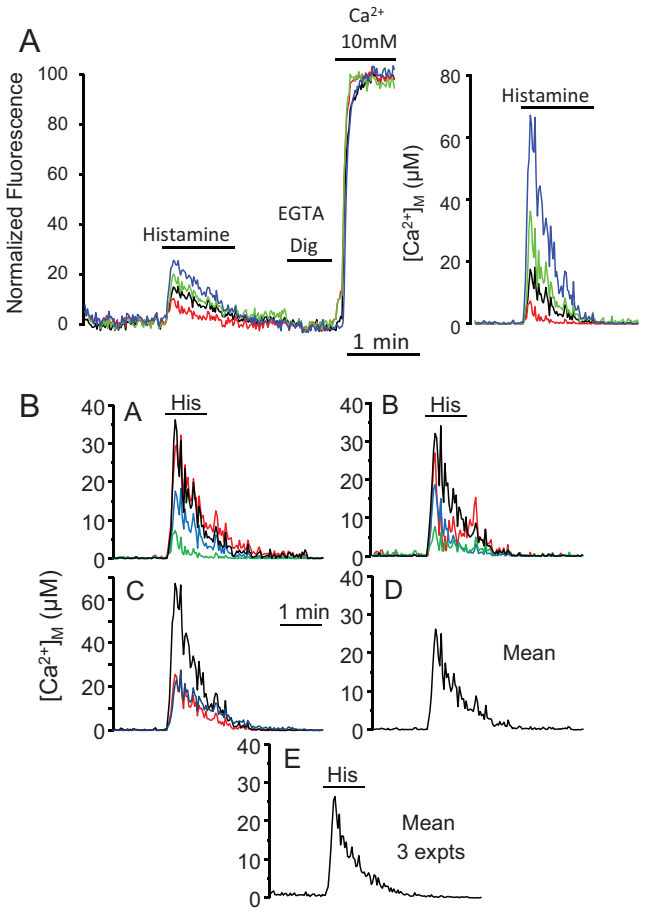

Fig. 4. Histamine-induced $[Ca^{2+}]_M$ peaks in single HeLa cells measured with rhod-5N. 100 μM histamine was added as indicated to rhod-5N loaded HeLa cells. Then, cells were permeabilized and the calibration solution containing 10 mM $[Ca^{2+}]$ was added to allow calibration. (a) How the changes in normalized fluorescence translate into $[Ca^{2+}]_M$ values when the calibration curve is applied. (b) A series of calibrated curves obtained in single cells of one experiment (panels A, B and C), the mean of that experiment (panel D) and the mean of 3 similar experiments (panel E).

is therefore higher than the in vitro one (320 μM), as occurs also with rhod-2 [6] and with ratiometric pericam [5].

We have then used this calibration to monitor $[Ca^{2+}]_M$ dynamics in experiments performed in intact and permeabilized HeLa cells loaded with rhod-5N. First, in permeabilized cells, the data shown in Figs. 2 and 3 indicate that perfusion of known $[Ca^{2+}]$ buffers triggers Ca^{2+} uptake by mitochondria reaching $[Ca^{2+}]_M$ values of hundreds of micromolar, as previously found using low-Ca^{2+}-affinity aequorin [3,8]. Ca^{2+}-release from mitochondria was strongly dependent on the $[Na^+]$ in the medium, as expected for a flux driven mainly by a Na^+/Ca^{2+} exchange mechanism, and collapse of the mitochondrial membrane potential with FCCP induced a rapid release of Ca^{2+} from mitochondria. Both the absolute $[Ca^{2+}]$ and the kinetics of all these processes were similar and consistent with those previously found using aequorin [8,10]. Therefore, these experiments show that $[Ca^{2+}]_M$ measurements obtained with low-Ca^{2+}-affinity aequorin and rhod-5N are fully consistent.

The advantage of rhod-5N over aequorin is that it allows making single-cell and subcellular imaging studies. Again here, the magnitude of the histamine-induced $[Ca^{2+}]_M$ peaks was similar on average to those previously measured with aequorin, around 20–30 μM [12,13]. However, the single-cell data showed an important degree of heterogeneity among the peaks obtained in different cells. We initially thought that this variability could be due to a parallel variability in the cytosolic $[Ca^{2+}]$ peaks ($[Ca^{2+}]_c$). However,

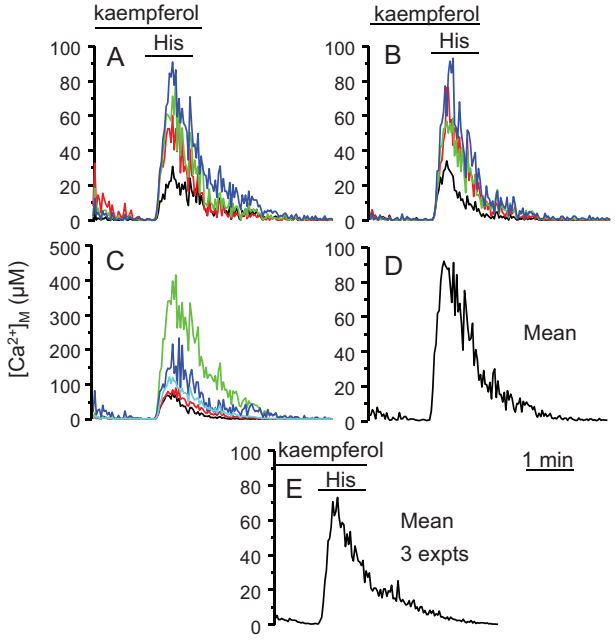

Fig. 5. Histamine-induced $[Ca^{2+}]_M$ peaks in the presence of kaempferol in single HeLa cells measured with rhod-5N. Experimental details as in Fig. 4, but cells were stimulated with histamine in the presence of 10 μM kaempferol, as indicated.

experiments designed to measure simultaneously cytosolic and mitochondrial $[Ca^{2+}]$ (using cells loaded with both fura-2 and rhod-5N) showed that there was little correlation among the cytosolic and mitochondrial $[Ca^{2+}]$ peaks. There may be several explanations for this finding, either variations in mitochondrial Ca^{2+} uptake ability among different cells or perhaps more probably single-cell variations in the functionality of the close contacts between ER and mitochondria, the mitochondria associated ER membrane or MAM

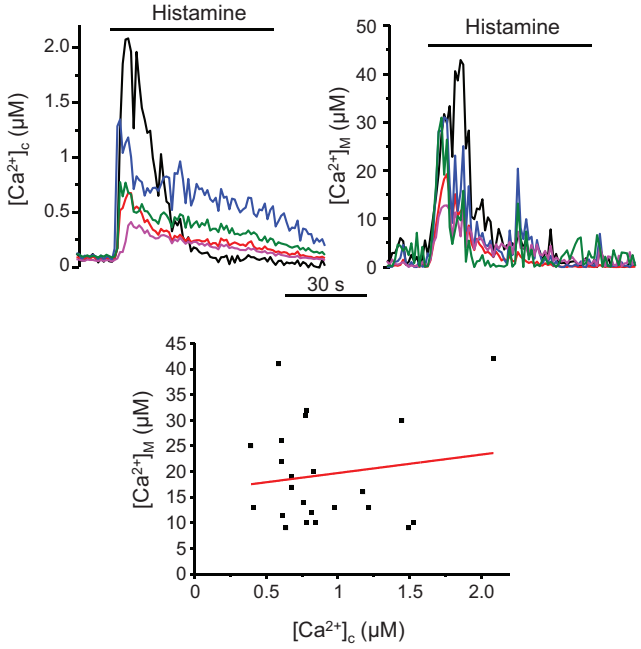

Fig. 6. Parallel measurement of cytosolic and mitochondrial $[Ca^{2+}]$ peaks in single HeLa cells. HeLa cells loaded with both fura-2 and rhod-5N were imaged as described in Section 2 to monitor both $[Ca^{2+}]_c$ and $[Ca^{2+}]_M$ simultaneously. The upper panel shows the peaks obtained in both compartments in 5 cells from the same experiment. The lower panel shows the relationship among the cytosolic and mitochondrial $[Ca^{2+}]$ peaks in 23 single cells obtained from 5 similar experiments.

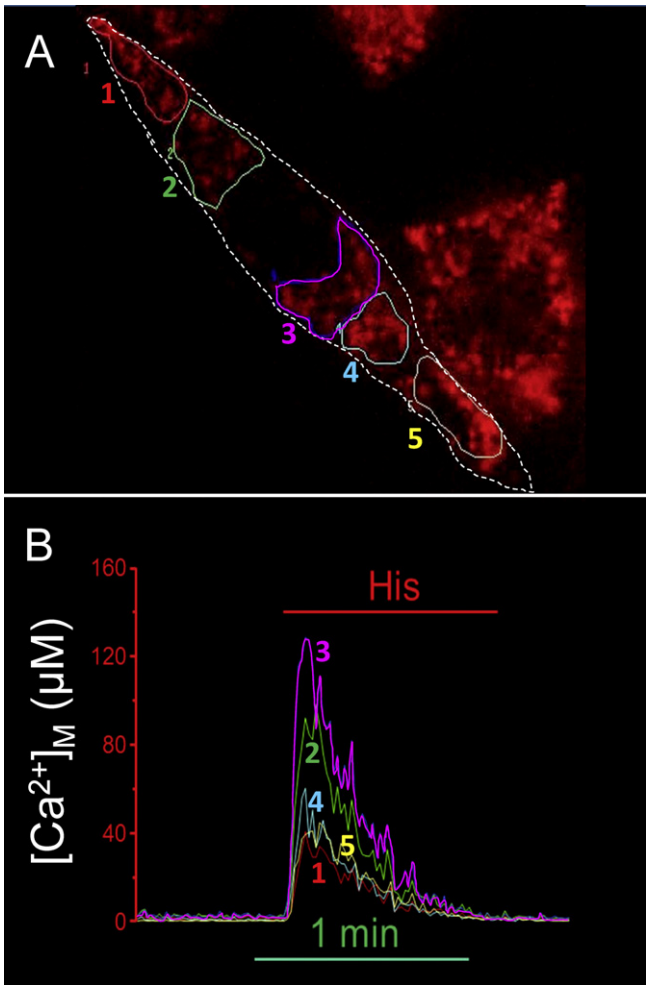

Fig. 7. Histamine-induced $[Ca^{2+}]_M$ peaks in subcellular regions of single HeLa cells measured with rhod-5N. Experiments were performed as indicated in Fig. 4, but monitoring subcellular regions. The upper panel shows the regions studied in a typical cell and the final rhod-5N fluorescence obtained after perfusion of the 10 mM $[Ca^{2+}]$ calibration medium. The lower panel shows the histamine-induced $[Ca^{2+}]_M$ peaks obtained in each region of this cell.

[20]. In fact, the Ca^{2+}-transfer between ER and mitochondria has been shown to be critically dependent on the gap between both organelles [16]. If this hypothesis is correct, the size of the $[Ca^{2+}]_M$ peak would depend more on the spatial relationship between mitochondria and endoplasmic reticulum than on the amount of Ca^{2+} actually released.

The variability was still present when the histamine induced $[Ca^{2+}]$ peaks were obtained in the presence of the uniporter activator kaempferol, although the mean $[Ca^{2+}]_M$ peak in the presence of kaempferol was about 3–4-fold larger and the range of the peak magnitudes went from 30 to 400 μM. The mean $[Ca^{2+}]_M$ peak values obtained in the presence of kaempferol were again similar to those previously found with aequorin [13]. Finally, the data obtained at the subcellular level show also significant variability in the $[Ca^{2+}]_M$ responses within every single cell. This variability had been already predicted in the initial aequorin studies in HeLa cells, when it was shown that repetitive stimulation induced aequorin consumption only in a selected mitochondrial population [21]. Further evidence for this phenomenon was later obtained in other cells [3] and it has been interpreted on the basis of the presence of local Ca^{2+} microdomains generated in regions of close apposition of mitochondria and ER, or mitochondria and the plasma membrane. Direct evidence for the presence of these cytosolic $[Ca^{2+}]$ microdomains in the vicinity of mitochondria has been recently provided [16,22]. Our data show also that the larger $[Ca^{2+}]_M$ increases occur in cell regions close to the nucleus. This is consistent with the data of Giacomello et al. [22], which also show a larger accumulation of mitochondria-associated Ca^{2+} microdomains close to the nucleus.

In conclusion, we show in this paper that the very low-Ca^{2+}-affinity dye rhod-5N is able to measure the same high $[Ca^{2+}]_M$ values previously obtained using aequorin and provides new data on the behavior of $[Ca^{2+}]_M$ at the single-cell and subcellular level. Therefore, we find that the $[Ca^{2+}]_M$ values obtained with fluorescent dyes and targeted proteins are consistent if the probes have similar Ca^{2+}-affinity. Although rhod-5N still has the same problems reported for rhod-2 during repetitive stimulation [9], it has the advantage of being able to monitor high levels of $[Ca^{2+}]$, to obtain single-cell and subcellular resolution, and to allow easy simultaneous measuring of $[Ca^{2+}]_c$ and $[Ca^{2+}]_M$ in single cells.

Conflict of interest

The authors certify that there are no conflicts of interest of any kind regarding the manuscript.

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