Monitoring mitochondrial [Ca^{2+}] dynamics with rhod-2, ratiometric pericam and aequorin

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Summary

The dynamics of mitochondrial [Ca^{2+}] ([Ca^{2+}]$_M$) plays a key role in a variety of cellular processes. The most important methods available to monitor [Ca^{2+}]$_M$ are fluorescent dyes such as rhod-2 and specifically targeted proteins such as aequorin or pericam. However, significant discrepancies, both quantitative and qualitative, exist in the literature between the results obtained with different methods. We have made here a systematic comparison of the response of several fluorescent dyes, rhod-2 and rhod-FF, and two Ca^{2+}-sensitive proteins, aequorin and pericam. Our results show that measurements obtained with aequorin and pericam are consistent in terms of dynamic Ca^{2+} changes. Instead, fluorescent dyes failed to follow Ca^{2+} changes adequately, especially during repetitive stimulation. In particular, measures obtained with rhod-2 or rhod-FF evidenced the previously reported Ca^{2+}-dependent inhibition of mitochondrial Ca^{2+} uptake, but data obtained with aequorin or pericam under the same conditions did not. The reason for the loss of response of fluorescent dyes is unclear. Loading with these dyes produced changes in mitochondrial morphology and membrane potential, which were small and reversible at low concentrations (1-2μM), but produced large and prolonged damage at higher concentrations. **In addition, cells loaded with low concentrations of rhod-2 suffered large changes in mitochondrial morphology after light excitation.** Our results suggest that [Ca^{2+}]$_M$ data obtained with these dyes should be taken with care.

Introduction

The dynamics of $[Ca^{2+}]$ in the mitochondrial matrix has received much attention in the last 20 years because of its importance in a large variety of critical cellular processes, from energy production to apoptosis, going through the regulation of many cytosolic Ca^{2+}-dependent processes [1-5]. Measurements of mitochondrial $[Ca^{2+}]$ have been made using different methods, which can be classified in two categories: fluorescent Ca^{2+}-sensitive dyes such as rhod-2 or similar, and luminescent and fluorescent targeted proteins such as aequorin, pericam or camaleons. Unfortunately, data obtained with each of these approaches are very different, both qualitatively and quantitatively, and the reasons for the discrepancies are still unclear. On the first place, large differences in the maximum free $[Ca^{2+}]$ that can be attained in the mitochondrial matrix have been reported. While studies using fluorescent dyes report maximum $[Ca^{2+}]_M$ values of 2-3μM [6-9], data obtained with targeted luminescent and fluorescent proteins indicate that $[Ca^{2+}]_M$ can reach much higher values, up to tenths or hundreds of micromolar [10-14]. In a recent paper, we discussed this point and provided new evidence in favour of the correctness of the measurements obtained with targeted proteins, particularly aequorin [15].

On the other hand, the discrepancies between the measurements of $[Ca^{2+}]_M$ with dyes or targeted proteins are also qualitative in some cases. This means that the difference is not only a change in scale. Instead, significant changes in the behaviour or kinetics of $[Ca^{2+}]_M$ appear when comparing measurements obtained with both kinds of methods. This kind of comparison has never been made systematically, but some clear discrepancies can be easily found in the literature, particularly regarding the effect of repetitive stimulations. For example, Collins et al. [6] found using rhod-2 to measure $[Ca^{2+}]_M$ that a second application of histamine to HeLa cells produced the same $[Ca^{2+}]$ response in the cytosol, but a much smaller $[Ca^{2+}]_M$ response. However, Filippin et al.

[13], using mitochondrially-targeted pericam in the same cells, found that both the cytosolic and the mitochondrial [Ca^{2+}] responses were completely preserved during repetitive stimulation. Similarly, Moreau et al. [16], using permeabilized RBL-1 cells loaded with rhod-2, found that the response to a second stimulation with Ca^{2+} of permeabilized RBL cells was much smaller than the first one, concluding that Ca^{2+} inactivates the mitochondrial Ca^{2+} uniporter. This finding was consistent with that of Collins et al., 2001 obtained with rhod-2, mentioned above, but not with that of Filippin et al. [13], obtained with pericam. In addition, data obtained with low-affinity targeted aequorin in several types of cells argue also against the presence of such inactivation of the mitochondrial Ca^{2+} uniporter by Ca^{2+}. In chromaffin cells, repetitive stimulation with high-K^+ depolarization or caffeine triggered repetitive and similar increases in mitochondrial [Ca^{2+}] [10]. Similarly, addition of Ca^{2+} to permeabilized HeLa cells produced a long-lasting increase in $[Ca^{2+}]_M$ with no signs of inactivation [15]. In conclusion, the differential behaviour of fluorescent dyes and targeted proteins when measuring mitochondrial [Ca^{2+}] may actually lead to a different description of physiological phenomena and it is therefore necessary to make a more systematic evaluation of the measurements obtained by both methods.

We have made here parallel measurements of $[Ca^{2+}]_M$ using rhod-2 and the targeted proteins aequorin and pericam. Our results confirm that aequorin and pericam show qualitatively similar $[Ca^{2+}]_M$ measurements in HeLa cells, but measurements obtained with rhod-2 deviate from the response obtained with the other probes, particularly during repetitive stimulation.

Methods

Cell culture and targeted aequorin expression.

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. The constructs for cytosolic aequorin and for mutated aequorin targeted to mitochondria have been described previously [10,17]. The construct for mitochondrially targeted pericam (2mt8PR, see ref. 18) was kindly supplied by Dr. María Cano-Abad, Hospital Universitario de La Princesa, Madrid. Transfections were carried out using Metafectene (Biontex, Munich, Germany). The construct for mitochondrially-targeted EGFP was prepared by inserting the 37 aminoacids mitochondrial presequence from subunid viii of cytochrome oxidase [19] before the EGFP sequence.

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

HeLa cells were plated onto 13 mm round coverslips and transfected with the plasmid for either cytosolic aequorin (cytAEQ) or mitochondrially-targeted mutated aequorin (mitmutAEQ). For aequorin reconstitution, HeLa cells were incubated for 1-2h at room temperature in standard medium (145mM NaCl, 5mM KCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 10mM glucose, and 10mM HEPES, pH 7.4) with 1μM of coelenterazine wild type or coelenterazine n, as indicated. After reconstitution, cells were placed in the perfusion chamber of a purpose-built luminometer and perfused with the required solutions. To perform experiments in permeabilized cells, cells were first perfused for 1 min with standard medium containing 0.5mM EGTA instead of Ca^{2+}, followed by 1 min of intracellular medium (130mM KCl, 10mM NaCl, 1mM $MgCl_2$, 1mM potassium phosphate, 0.5mM EGTA, 1mM ATP, 20μM ADP, 10mM L-malate, 10mM glutamate, 10mM succinate, 20mM Hepes, pH 7) containing 100μM digitonin. 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 some experiments, temperature was set at 22ºC 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 [12,20]. Statistical data are given as mean±S.E.M.

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

HeLa cells were plated onto 12 mm round coverslips, transfected with the plasmid for mitochondrially-targeted ratiometric pericam (mitRP) and used after 48h for the experiments. Cells were mounted in a cell chamber in the stage of a Zeiss Axiovert 200 microscope under continuous perfusion. Single cell fluorescence was excited at 415 nm and 485 nm using a Cairn monochromator (200-300 ms excitation at each wavelength every 2s, 10nm bandwidth) and images of the emitted fluorescence obtained with a 60x objective were collected using a 505DCXR dichroic mirror and a HQ535/50m emission filter (both from Chroma Technology) and recorded by a Hamamatsu ORCA-ER camera. Single cell fluorescence records were ratioed using the Metafluor program (Universal Imaging). **Fig. 1 shows the traces obtained at both wavelengths and the ratio from a typical experiment performed with mitRP. In all the experiments performed, the increase in the ratio was mainly due to the decrease in the fluorescence excited at 415nm, combined with a small increase at the longer wavelength. These changes are the expected ones for a change in [Ca^{2+}], while changes in pH tend to modify the ratio mainly through the increase in the fluorescence obtained at the longer wavelength [13]. It has also been shown before that there is no change in mitochondrial pH during the histamine-induced mitochondrial [Ca^{2+}] peak [13].** Experiments were performed at 25ºC using an on-line heater from Harvard Apparatus.

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

HeLa cells were loaded with rhod-2 or rhod-FF by incubation in standard medium containing 1-10 μM (as indicated) of the acetoxymethyl ester derivative 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 of the microscope as above. Single cell fluorescence was excited at 545 nm using a Cairn monochromator (300ms excitation every 2s, 10nm bandwidth) and images of the emitted fluorescence obtained with a 60x 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 37ºC using an on-line heater from Harvard Apparatus.

Confocal studies.

Cells were imaged at room temperature on a Leica TCS SP2 confocal spectrophotometer using a 63x oil immersion objective. Mitochondrially-targeted EGFP (mitEGFP) was excited with the 488nm line of the Argon laser, and the fluorescence emitted between 500 and 600 nm was collected. To monitor mitochondrial membrane potential, cells were loaded with 100nM TMRE for 30 min at 37ºC and then washed 3 times with extracellular medium before the addition of rhod-2AM. Images were taken before and during the rhod-2 loading period using the excitation of the 488nm line of the Argon laser and measuring the fluorescence emitted between 500 and 700nm.

Materials.

Wild type coelenterazine, coelenterazine n, TMRE, rhod-2AM and rhodFF-AM were obtained from Molecular Probes, Or. U.S.A. or Biotium Inc., Hayward, Ca, U.S.A. Reversan, Ko143 and tempol were from Tocris, Bristol, UK. Other reagents were from Sigma, Madrid or Merck, Darmstadt.

Results

We have first addressed the discrepancy among the $[Ca^{2+}]_M$ measurements obtained with rhod-2 and pericam in the second response to histamine of HeLa cells, and we have added new data obtained with low-Ca^{2+}-affinity aequorin. Panel A of Fig. 2 shows data obtained with rhod-2 loaded cells, showing that the second response to histamine was clearly decreased with respect to the first one, as previously described by Collins et al. [6]. Panel B of the figure shows instead that using pericam to measure $[Ca^{2+}]_M$, the second peak was very similar to the first one, as previously described by Filippin et al. [13]. Panel C shows the same experiment performed using low-Ca^{2+}-affinity aequorin. Previous data of double histamine stimulation obtained in HeLa cells have always been obtained with high Ca^{2+}-affinity aequorin. Under those conditions, the heterogeneity in Ca^{2+} accumulation among different mitochondrial subpopulations induces a rapid consumption of aequorin during the first pulse in about 30% of the mitochondria, those that are apparently coupled to the release sites in the endoplasmic reticulum. Thus, the second pulse produces a much smaller response, but this is mainly due to the consumption of the probe in the high-responding mitochondria during the first pulse [13,21,22]. Now, using low-Ca^{2+}-affinity aequorin, we show that the peak of $[Ca^{2+}]_M$ induced by two consecutive histamine stimulations was identical. For the calculations of $[Ca^{2+}]$ in these experiments, we have assumed, according to previous data [22], that the relevant mitochondrial space was a 30% of the total. Thus, the traces shown reveal the $[Ca^{2+}]_M$ transient only in the mitochondrial population closer to the release sites from the ER. Loading the cells with rhod-2 did not modify the response measured with aequorin. Therefore, measurements with low-Ca^{2+}-affinity aequorin confirm the kinetics of the $[Ca^{2+}]_M$ changes data obtained with pericam.

Figure 3 shows the effect on $[Ca^{2+}]_M$ of repetitive addition of a Ca^{2+} buffer to permeabilized HeLa cells. In accordance with the data of Moreau et al., 2006, obtained

in permeabilized RBL cells, addition of two consecutive pulses of 10μM Ca^{2+} triggered also here very different responses when measured with rhod-2, the second being much smaller than the first one. Panel A shows two representative experiments. The same results were found when the lower Ca^{2+}-affinity rhod-FF was used instead of rhod-2 (panel B). However, when the same experiment was repeated under the same conditions in cells expressing either pericam (panel C) or aequorin (panel D) in their mitochondria, the results were very different. Both targeted proteins reported in fact nearly identical mitochondrial [Ca^{2+}] peaks induced by the first and the second [Ca^{2+}] pulse. In addition, loading the cells with rhod-2 did not modify the response obtained in the experiments performed with aequorin, showing that the discrepancies are not due to the presence of rhod-2 modifying the Ca^{2+}-response, but to an apparent lose of Ca^{2+}-sensitivity of rhod-2 along the experiment. **To investigate this point further, we have studied the effect of the superoxide scavenger tempol, and of the multidrug transporters inhibitors reversan and Ko143, in experiments of double stimulation with a 10μM [Ca^{2+}] buffer similar to those of Fig. 3, panel A. The results obtained for the relative height of the second [Ca^{2+}] peak with respect to the first one (in mean±s.e. of the percentage) were the following: control, 7.4%±0.6% (n=40 cells analyzed); reversan, 13%±0.5% (n=37 cells analyzed); Ko143, 10.4%±1.2% (n=27 cells analyzed); tempol, 14.5%±1.6% (n=27 cells analyzed). Therefore, neither reactive oxygen species nor rhod-2 extrusion through multidrug transporters appear to be involved in the lack of response of rhod-2.**

The data shown in the above figures point to the presence of problems of rhod-2 to adequately monitor the $[Ca^{2+}]_M$ dynamics under some circumstances, particularly during repetitive stimulation. However, the lack of Ca^{2+}-sensitivity of rhod-2 may sometimes appear even in the absence of previous stimulation. Moreau et al., 2006, reported that the protonophore FCCP completely abolishes the increase of

mitochondrial [Ca^{2+}] induced by 10-100μM [Ca^{2+}] pulses in permeabilized cells. Given the high Ca^{2+}-affinity of rhod-2 (**K_d inside mitochondria of 1.3μM**, see ref. 6), we found surprising that no Ca^{2+} increase could be detected in mitochondria under these conditions, as simple equilibration of cytosolic and mitochondrial [Ca^{2+}] by any pathway would rapidly lead to complete saturation of rhod-2. Fig. 4 shows measurements performed with aequorin indicating that Ca^{2+} enters in fact in mitochondria under these conditions, though slowly, and approaches equilibration with the [Ca^{2+}] in the buffer, as could be expected. Panels A and D in the figure show that addition of a 10μM Ca^{2+} buffer to permeabilized cells produces a very fast Ca^{2+} uptake into mitochondria both at 22ºC (panel A) or 37ºC (panel D), reaching [Ca^{2+}] of hundreds of micromolar in few seconds. Instead, in the presence of a protonophore, the rate of $[Ca^{2+}]_M$ increase was much lower, particularly at 22ºC (see panels B and C for the effect of 10, 20 and 100μM Ca^{2+} at 22ºC and panel E for the effect of 10μM Ca^{2+} at 37ºC), and approached equilibration with the [Ca^{2+}] in the buffer. That increase, however, should be enough to saturate mitochondrial rhod-2 with Ca^{2+}. Similarly, panel F shows that measurements in HeLa cells loaded with pericam and perfused with a 10μM Ca^{2+} buffer also show an increase in mitochondrial [Ca^{2+}] in the presence of FCCP. However, in accordance with the data of Moreau et al., 2006, measurements performed with rhod-2 showed no increase at all in fluorescence under these conditions, even after perfusion of 100μM Ca^{2+} (data not shown).

To investigate the origin of the discrepancies shown above, we have next studied if loading with rhod-2 produced changes in the mitochondrial morphology or in the responses of cytosolic or mitochondrial [Ca^{2+}] to stimuli. To study the mitochondrial morphology, HeLa cells expressing EGFP targeted to the mitochondria were used. Confocal images were taken before rhod-2 loading and then the dye was added at several concentrations (1-5μM) and the EGFP fluorescence was followed on-line

during the 30 min loading period and in some cases also during the subsequent dye washing period. Fig. 5 shows that addition of rhod-2AM was followed by a progressive and concentration-dependent fission of mitochondria, with rupture of the characteristic long mitochondrial tubules to generate round small mitochondria. This phenomenon was slow and limited at 1μM rhod-2AM, much more evident at 2μM and very fast and nearly complete at 5μM rhod-2AM (Fig. 5A). In addition, it was partially reversible after dye washing, particularly at low concentrations, as shown in Fig. 5B. Similar results were obtained using rhod-FF (data not shown).

Therefore, rhod-2AM loading clearly affected mitochondrial morphology, particularly at concentrations above 1μM. This effect was accompanied by a decrease in the stimulus-induced mitochondrial [Ca^{2+}] peaks if the rhod-2AM concentrations were above 2μM. Fig. 6 shows that loading with rhod-2AM or its low Ca^{2+}-affinity derivative rhodFF-AM up to 10μM produced no change in the cytosolic [Ca^{2+}] peak induced by histamine. Loading the cells with rhod-2AM or rhodFF-AM 2μM did not change either the mitochondrial [Ca^{2+}] peak induced by histamine. However, when cells were loaded with higher rhod-2AM or rhodFF-AM concentrations (5-10μM), a clear decrease in the mitochondrial [Ca^{2+}] peak induced by histamine was observed. The reason for these effects of high rhod concentrations could rely in the increased buffering capacity. However, the effects of high rhod concentrations on mitochondrial morphology reported above suggest that the decrease in the Ca^{2+} uptake rate they induce may also be due to mitochondrial damage. In fact, loading with 5 μM rhod-2AM induced a significant decrease in mitochondrial membrane potential. Fig. 7 shows that loading with 5μM rhod-2AM induced a large decrease in TMRE fluorescence measured by confocal microscopy during the 30 min loading period. In the absence of rhod-2AM, no significant changes in fluorescence were observed in similar periods (data not shown).

We have finally investigated if these effects of rhod-2 could be due to changes in the rhod-2 molecule induced by light excitation. We have used the epifluorescence illumination of the confocal microscope to give a brief (1 min) continuous illumination (through a BP 515-560 filter) to HeLa cells expressing mit-EGFP and loaded or not with 1μM rhod-2AM. We have obtained confocal images of the cells just before and at several times after the illumination, and we have observed that the cells loaded with rhod-2 (but not the controls) underwent a complete granulation of their mitochondria few minutes after illumination. Fig. 8 shows confocal images taken before and 1 or 5 min after the illumination. While no changes were observed in the control cells, those loaded with rhod-2 underwent a progressive mitochondrial granulation which was still not apparent 1 min after illumination but was nearly complete 5 min later. No further changes in morphology were observed during the following 20 min (data not shown). In conclusion, illumination of rhod-2 loaded cells induces important changes in mitochondrial morphology which may be responsible of the differential behaviour of this probe with respect to aequorin or pericam.

Discussion

We have made here a comparison of the ability to monitor the mitochondrial [Ca^{2+}] of several fluorescent dyes, including rhod-2, rhod-FF, one fluorescent targeted protein, pericam, and one luminescent targeted protein, aequorin. As mentioned above, large discrepancies have been reported among the $[Ca^{2+}]_M$ values measured with rhod-2 or other high Ca^{2+}-affinity dyes such as furaFF or fura6F [6-9], always below 2-3μM, and the much higher values measured by low-Ca^{2+}-affinity targeted proteins, either aequorin, pericam or cameleons [10-14]. **In part, the discrepancies may be explained by the different Ca^{2+}-affinity of the probes, which should lead to much faster saturation with Ca^{2+} of the high-affinity ones. In particular, rhod-2 has been shown to have an "in situ" mitochondrial Kd of 1.3μM [6], while the "in situ" mitochondrial Kd of pericam is nearly 10-fold larger, 11μM [13], and low-Ca^{2+}-affinity aequorin is able to measure $[Ca^{2+}]_M$ reliably up to the millimolar range [15]. However,** we show now that the discrepancies are also of a qualitative nature in some cases and reveal that under some circumstances, rhod-2 may undergo an apparent lose of Ca^{2+}-sensitivity.

Our data are consistent with previous data of other authors using rhod-2 to measure mitochondrial [Ca^{2+}], which showed that either a second stimulation with an agonist in intact HeLa cells or a second perfusion of a Ca^{2+} buffer in permeabilized cells produced always a much smaller increase in mitochondrial [Ca^{2+}] than the first one [6,16]. We have also reproduced data obtained with pericam indicating that two consecutive stimulations of HeLa cells with an agonist produce similar $[Ca^{2+}]_M$ peaks [13], and we have confirmed them by obtaining the same results with low-Ca^{2+}-affinity aequorin. In addition, we have shown that both pericam and aequorin measure identical increases in $[Ca^{2+}]_M$ during repetitive perfusions of Ca^{2+} buffers in

permeabilized cells. The reason for the discrepancies does not rely in the Ca^{2+}-buffering capacity of rhod-2, as loading with this dye at the usual concentrations (1-2μM) did not modify mitochondrial Ca^{2+} dynamics measured with aequorin. When the concentration of rhod-2AM or rhodFF-AM was increased during loading to 5 or 10μM, the mitochondrial [Ca^{2+}] peaks measured with aequorin were in fact decreased. However, although this effect could in part be attributed to the increased buffering in the presence of the chelator, it could also be due to other reasons, as these concentrations induced important changes in the mitochondrial morphology and a decrease in mitochondrial membrane potential.

It was also very surprising to find that rhod-2 was unable to detect the increase in $[Ca^{2+}]_M$ that occurs when 10-100μM Ca^{2+} buffers are perfused in the presence of protonophores [16], an experimental condition which leads to equilibration of $[Ca^{2+}]_M$ with the [Ca^{2+}] present in the buffer, as it was easily detected by both aequorin and pericam. In this case the dye is actually missing the first increase in $[Ca^{2+}]_M$. The reason for this differential behaviour of rhod-2 and the other measuring methods is obscure. We have studied the effect of rhod-2 loading on mitochondrial architecture and on the $[Ca^{2+}]_M$ peaks induced by an agonist and we have found that concentrations of 5μM or more of rhod-2AM during loading produced a large impact on mitochondrial morphology, rupturing the mitochondrial tubules into small vesicles and decreasing the size of the mitochondrial [Ca^{2+}] peaks. Concentrations of 1-2μM, the ones used by us and many other authors, produced no effect on the mitochondrial [Ca^{2+}] peaks measured with aequorin and smaller and reversible effects on mitochondrial structure. **However, large changes in mitochondrial morphology were observed when cells loaded with low rhod-2AM concentrations were subjected to a brief period of illumination. Therefore, rhod-2 appears to be very sensitive to light excitation, generating products which lead to mitochondrial vesiculation.**

In summary, our results show that some Ca^{2+}-sensitive dyes widely used to measure mitochondrial [Ca^{2+}], such as rhod-2 and rhod-FF, have a response to dynamic [Ca^{2+}] changes in mitochondria which is qualitatively different to that of aequorin or pericam, at least in HeLa cells. The reason for this behaviour is unclear. It is not due to the effect of loading nor to the increased buffering, at least at the usual concentrations used for loading (1-2μM)**, but appears rather to be due to the generation of rhod-2 derivatives during light excitation, which are able to disorganize mitochondrial structure**. Therefore, results obtained with these dyes should be taken with care and confirmed with other methods. In particular, we have been unable to detect in HeLa cells any Ca^{2+}-inhibition of mitochondrial Ca^{2+} uptake when using either aequorin or pericam to monitor $[Ca^{2+}]_M$, although experiments performed with rhod-2 confirmed the results reported by Moreau et al, 2006. Our results here are consistent with previous data showing that continuous perfusion of Ca^{2+} buffers in permeabilized HeLa cells produces a prolonged increase in $[Ca^{2+}]_M$ with no signs of inactivation [15], and they are also consistent with data in intact HeLa cells and chromaffin cells showing that consecutive stimulations produce $[Ca^{2+}]_M$ peaks of similar magnitude [10,13].

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Figure Legends

Fig. 1. Monitorization of $[Ca^{2+}]_M$ with mitRP. HeLa cells expressing mitRP were stimulated with 100μM histamine as indicated. The traces show a typical single cell time course of the fluorescence obtained at 415nm and 485nm excitation, as indicated. The higher trace shows the ratio F485nm/F415nm.

Fig. 2. Effects of two consecutive agonist stimulations on $[Ca^{2+}]_M$ measured with rhod-2, aequorin or pericam. A. Hela cells were loaded with 1μM rhod-2 AM and then stimulated with 100μM histamine as indicated. Each trace is the mean of all the cells present in the microscope field (13 and 16) in two different experiments. These traces are representative of the results obtained in 22 similar experiments. B. Hela cells expressing mitRP were stimulated with 100μM histamine as indicated. The upper traces correspond to measurements obtained in three different single cells. The lower trace is the mean of all the cells present in the microscope field (16 cells). This experiment is representative of 23 similar ones. C. Hela cells expressing mitmutAEQ and reconstituted with coelenterazine n were stimulated with 100μM histamine as indicated. In the lower trace, cells were loaded with 2μM rhod-2AM prior to the experiment. The inset shows statistical data of the mean value of the first and second $[Ca^{2+}]_M$ peak obtained either in control cells (C1 and C2, n=11) or in cells loaded with rhod-2 (R1 and R2, n=7).

Fig. 3. Effects of two consecutive Ca^{2+} additions in permeabilized cells on $[Ca^{2+}]_M$ measured with rhod-2, rhodFF, aequorin or pericam. A. Hela cells were loaded with 1μM rhod-2AM and then permeabilized and stimulated twice with a 10μM $[Ca^{2+}]$ buffer as indicated. As in the rest of the panels of this figure, EGTA-containing intracellular medium was perfused before and in the intervals between Ca^{2+} applications. The trace

shown is the mean response of 20 cells present in the microscope field and is representative of 11 similar experiments. B. Hela cells were loaded with 2μM rhodFF-AM and then permeabilized and stimulated twice with a 10μM [Ca^{2+}] buffer as indicated. The trace shown is the mean response of 12 cells present in the microscope field and is representative of 7 similar experiments. C. Hela cells expressing mitRP were permeabilized and stimulated twice with a 10μM [Ca^{2+}] buffer as indicated. The trace shown is the mean response of 22 cells present in the microscope field and is representative of 10 similar experiments. D. Hela cells expressing mitmutAEQ and reconstituted with coelenterazine n were permeabilized and stimulated twice with a 10μM [Ca^{2+}] buffer as indicated. In the right panel, cells were loaded with 2μM rhod-2AM prior to the experiment. These experiments are representative of 3 similar ones of each type. Temperature was 22ºC.

Fig. 4. Effects of Ca^{2+} addition to permeabilized cells after mitochondrial depolarization. Hela cells expressing mitmutAEQ and reconstituted with coelenterazine were permeabilized and stimulated with 10, 20 or 100μM [Ca^{2+}] buffers in the presence or in the absence of 2μM FCCP, as indicated. Temperature was 22ºC in panels A, B and C, and 37ºC in panels D and E. The experiments shown are representative of 3-4 similar ones of each type. In panel F, Hela cells expressing mitRP were permeabilized and stimulated with a 10μM [Ca^{2+}] buffer in the presence of 2μM FCCP. The trace shown is the mean response of 15 cells and is representative of 4 similar experiments.

Fig. 5. Changes in mitochondrial morphology induced by rhod-2 loading. Hela cells expressing mitEGFP were imaged with a confocal microscope before and at different times during rhod-2 loading. In panel A, the effects of loading with 1 or 5μM rhod-2AM are shown. In panel B, cells were loaded with 2μM rhod-2AM for 30 min and then the

dye was washed and further images were taken 6, 25 and 40 min after that. The experiments shown are representative of 7-17 similar experiments of each type.

Fig. 6. Effect of loading with rhod-2 or rhod-FF on agonist-induced cytosolic and mitochondrial [Ca^{2+}] peaks. In the upper panel, Hela cells expressing cytAEQ were reconstituted with coelenterazine, loaded with different concentrations of rhod-2AM or rhodFF-AM and then stimulated with 100μM histamine to measure the cytosolic [Ca^{2+}] peak. In the lower panel, Hela cells expressing mitmutAEQ were reconstituted with coelenterazine, loaded with different concentrations of rhod-2-AM or rhodFF-AM and then stimulated with 100μM histamine to measure the mitochondrial [Ca^{2+}] peak. The insets on the right shows statistical data (mean ± s.e.m.) of several similar experiments of each type (number of experiments indicated over each bar). Significance was obtained by the ANOVA test (*, $p<0,05$; **, $p<0,005$; ***, $p<0,0005$).

Fig. 7. Effect of rhod-2 loading on mitochondrial membrane potential measured with TMRE. Hela cells were loaded with 100nM TMRE and imaged in a confocal microscope. Then, 5μM rhod-2AM was added and images were taken 5, 15 and 30 min after that. Mean fluorescence intensity was 78%, 58% and 47% of the initial one after 5, 15 and 30 min of rhod-2 loading, respectively. This experiment is representative of 10 similar ones.

Fig. 8. Effect of illumination and rhod-2 loading on mitochondrial morphology. HeLa cells expressing mitEGFP and loaded or not with 1μM rhod-2AM were imaged with a confocal microscope. After taking an initial image (panel A, control; panel D, rhod-2 loaded), cells were epi-illuminated for 1 min through a bandpass 515-560nm filter. Then, two additional images were taken 1 min (panel B, control; panel E, rhod-2 loaded) and 5 min (panel C, control; panel F, rhod-2 loaded) after

the end of the illumination period. The images shown are representative of the results obtained in 6 experiments with control cells and 10 experiments with rhod-2 loaded cells.

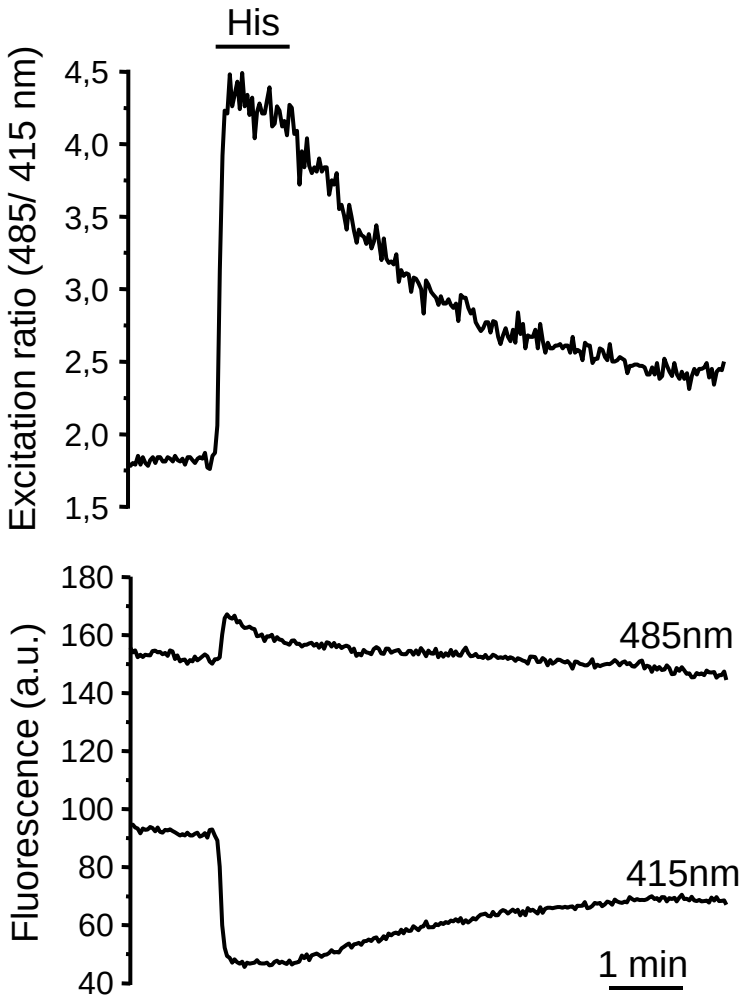
His
Excitation ratio (485/ 415 nm)
4,5
4,0
3,5
3,0
2,5
2,0
1,5
Fluorescence (a.u.)
180
160
140
120
100
80
60
40
485nm
415nm
1 min

Fig 1

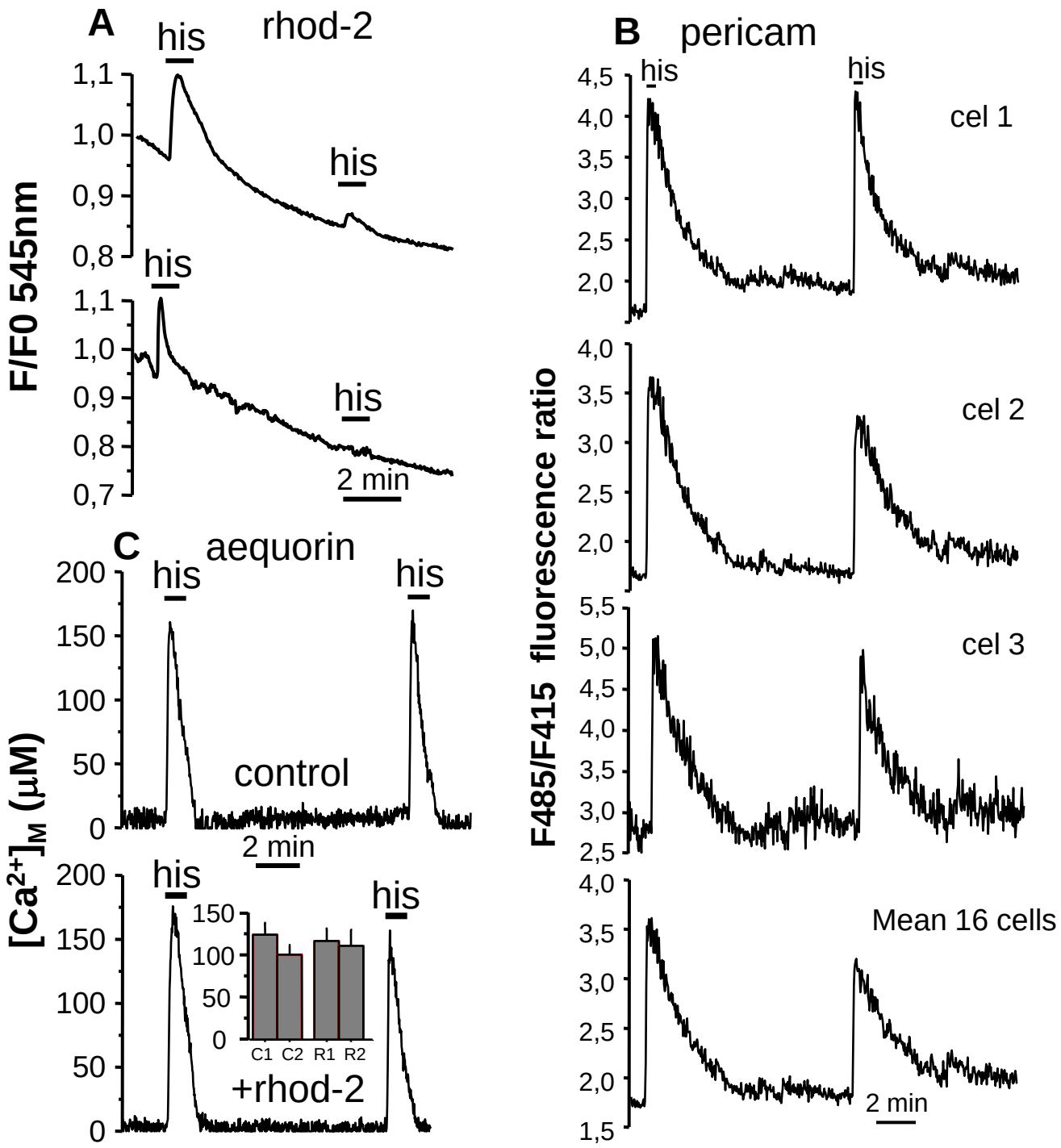
A
rhod-2
his
his
his
his
F/F0 545nm
1,1
1,0
0,9
0,8
0,7
2 min
B
pericam
his
his
cel 1
cel 2
cel 3
Mean 16 cells
F485/F415 fluorescence ratio
4,5
4,0
3,5
3,0
2,5
2,0
5,5
1,5
2 min
C
aequorin
his
his
control
2 min
his
his
+rhod-2
[Ca2+]M (μM)
200
150
100
50
0
C1 C2 R1 R2

Fig 2

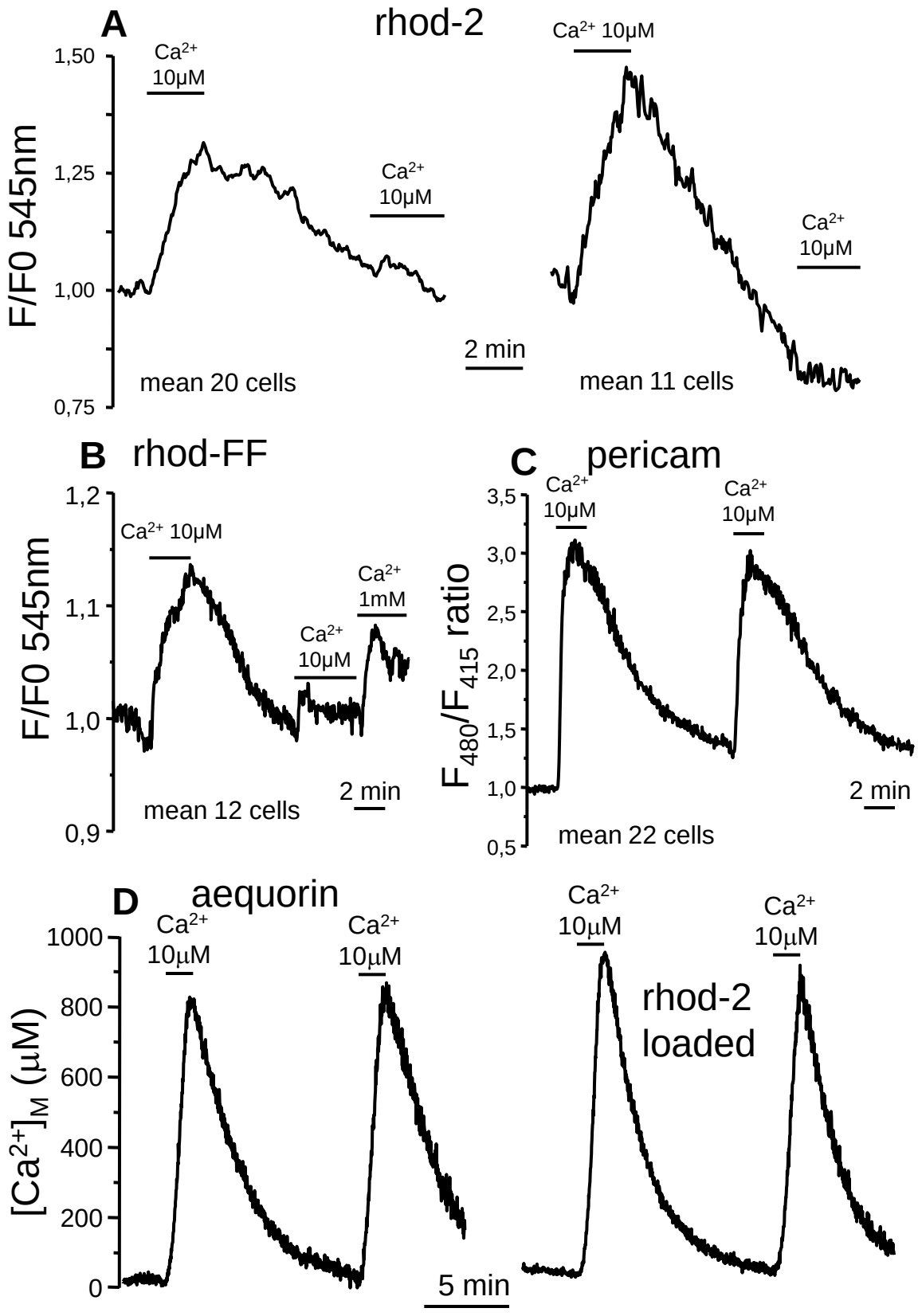
A
rhod-2
F/F0 545nm
1,50
1,25
1,00
0,75
Ca2+ 10μM
Ca2+ 10μM
Ca2+ 10μM
Ca2+ 10μM
2 min
mean 20 cells
mean 11 cells
B
rhod-FF
F/F0 545nm
1,2
1,1
1,0
0,9
Ca2+ 10μM
Ca2+ 1mM
Ca2+ 10μM
2 min
mean 12 cells
C
pericam
F480/F415 ratio
3,5
3,0
2,5
2,0
1,5
1,0
0,5
Ca2+ 10μM
Ca2+ 10μM
2 min
mean 22 cells
D
aequorin
[Ca2+]M (μM)
1000
800
600
400
200
0
Ca2+ 10μM
Ca2+ 10μM
Ca2+ 10μM
Ca2+ 10μM
rhod-2 loaded
5 min

Fig 3

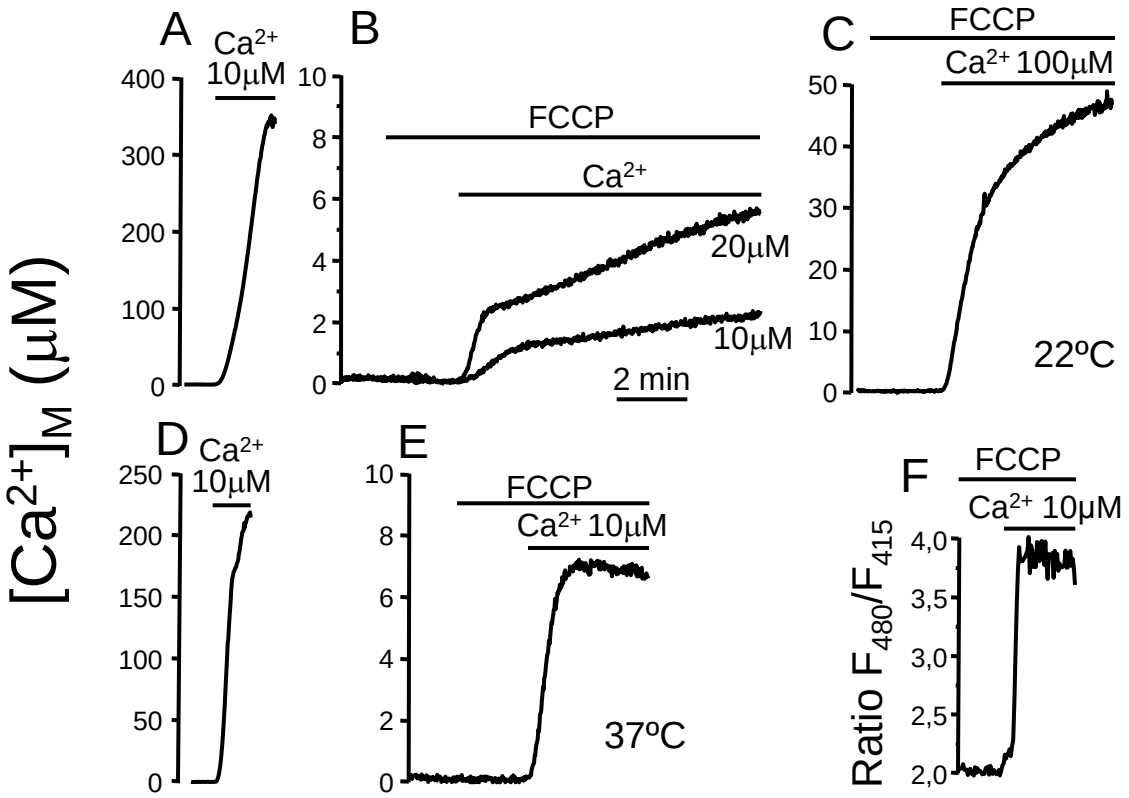
A
Ca2+
10µM
400
300
200
100
0
B
FCCP
Ca2+
20µM
10µM
2 min
10
8
6
4
2
0
C
FCCP
Ca2+ 100µM
50
40
30
20
10
0
22ºC
[Ca2+]M (µM)
D
Ca2+
10µM
250
200
150
100
50
0
E
FCCP
Ca2+ 10µM
37ºC
F
FCCP
Ca2+ 10µM
Ratio F480/F415
4,0
3,5
3,0
2,5
2,0

Fig 4

A

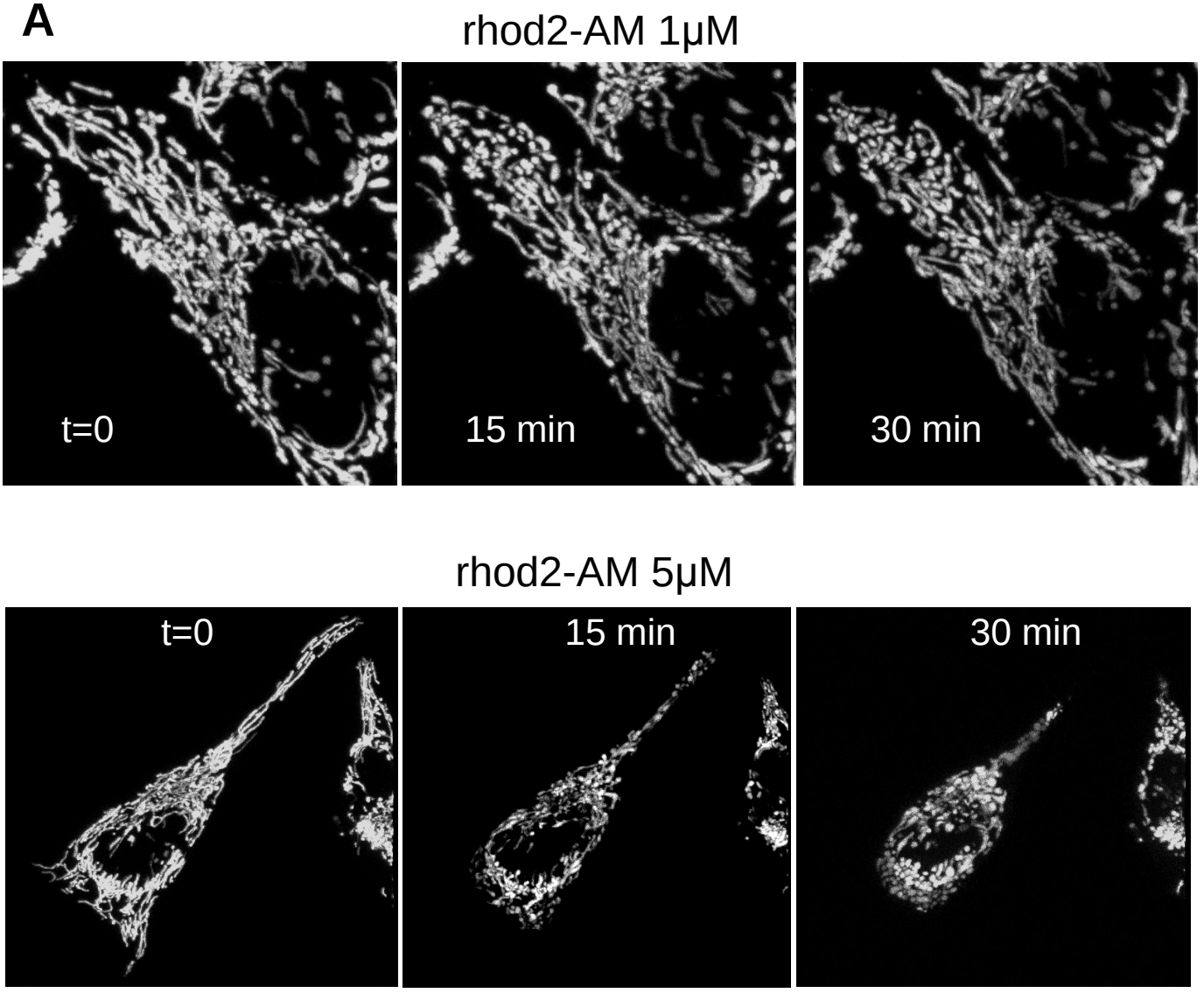

Fig 5A

B

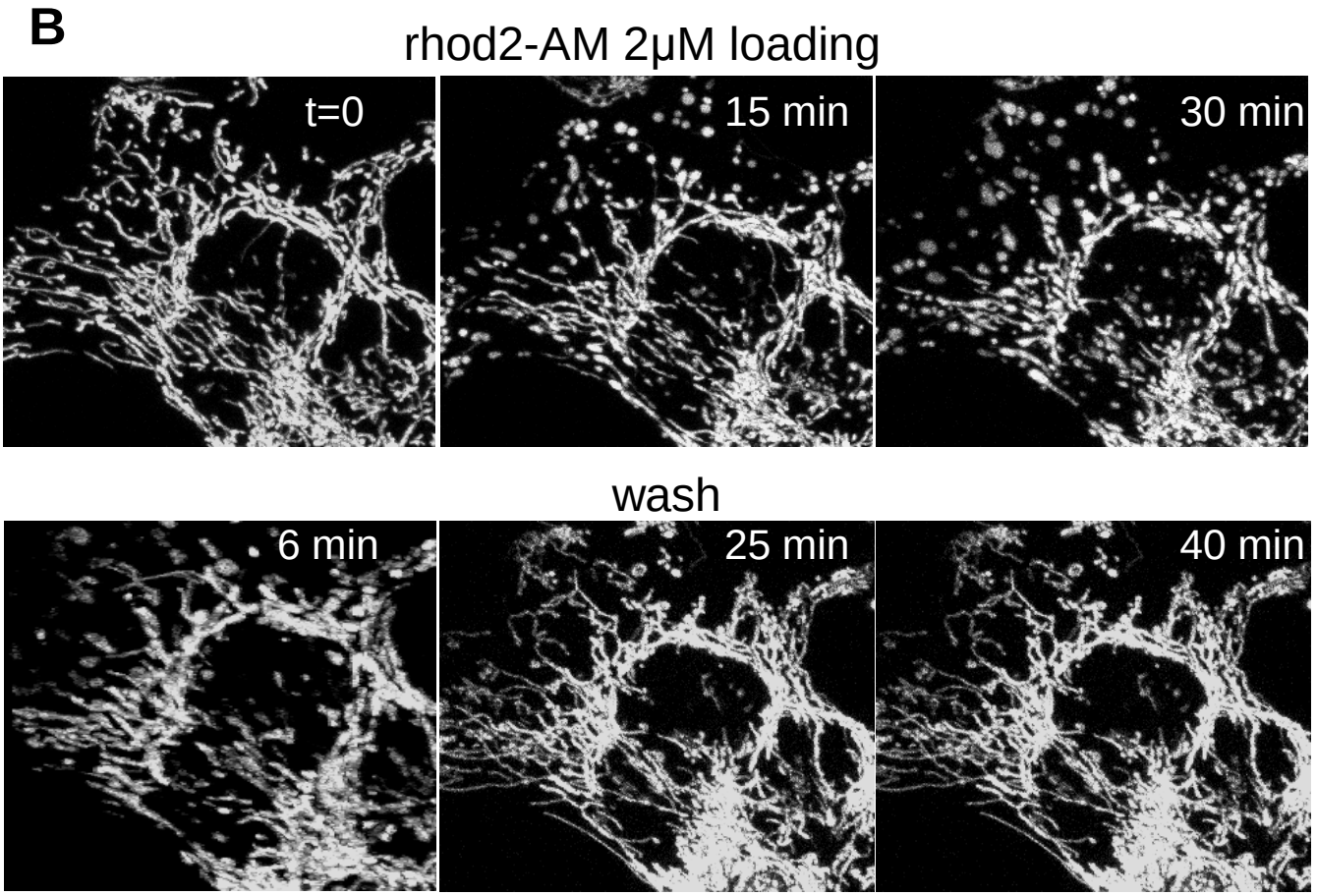

Fig 5B

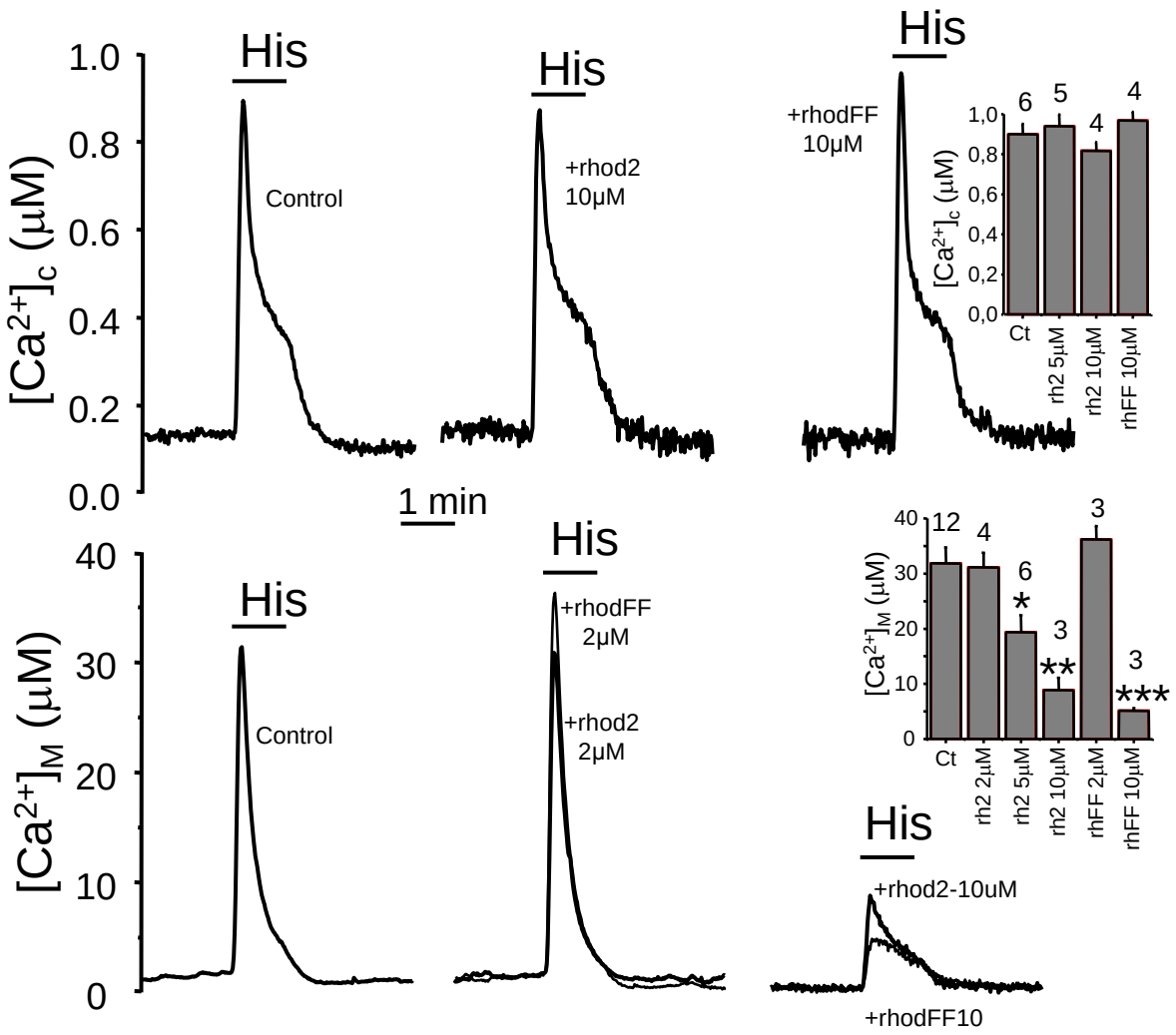
His
His
Control
+rhod2
10μM
1 min
[Ca2+]c (μM)
1.0
0.8
0.6
0.4
0.2
0.0
His
+rhodFF
10μM
6
5
4
4
1,0
0,8
0,6
0,4
0,2
0,0
[Ca2+]c (μM)
Ct
rh2 5μM
rh2 10μM
rhFF 10μM
His
His
Control
+rhodFF
2μM
+rhod2
2μM
[Ca2+]M (μM)
40
30
20
10
0
12
4
6
3
3
3
*
**
***
[Ca2+]M (μM)
Ct
rh2 2μM
rh2 5μM
rh2 10μM
rhFF 2μM
rhFF 10μM
His
+rhod2-10uM
+rhodFF10

Fig 6

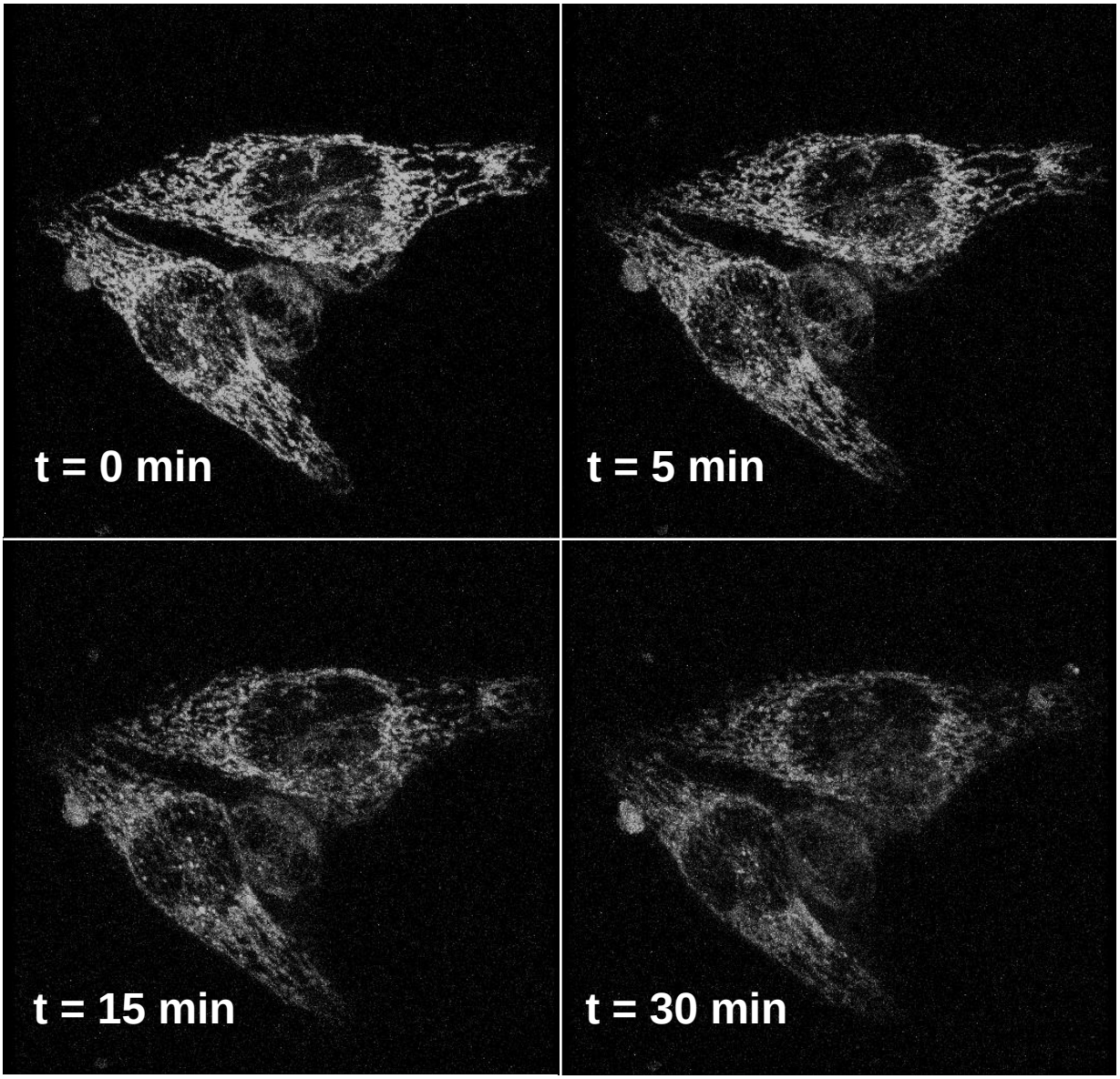

Fig 7

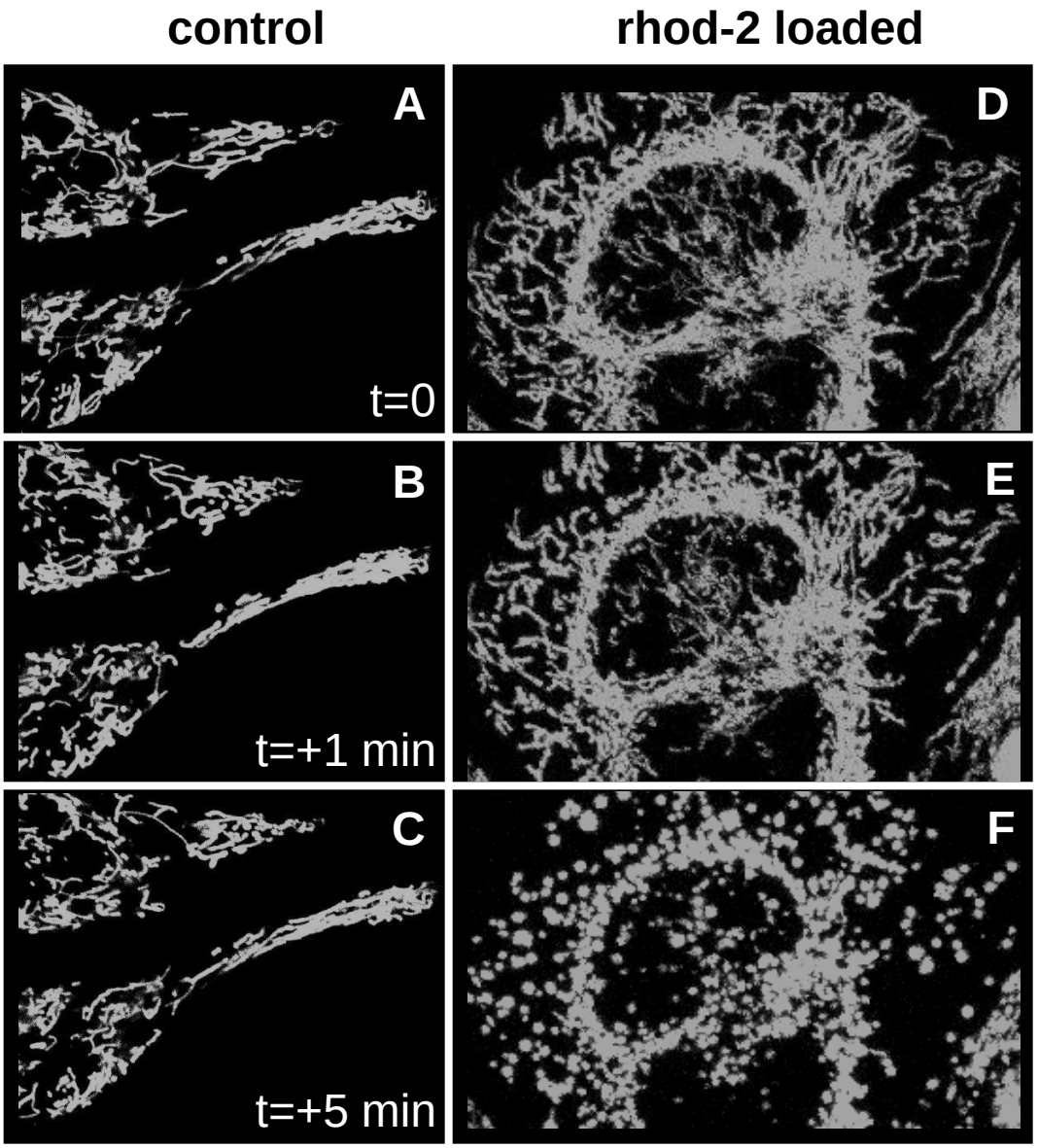

Fig 8

***Co-author Approval Statement**

I can certify that all authors of the manuscript entitled “Monitoring mitochondrial [Ca^{2+}] dynamics with rhod-2, ratiometric pericam and aequorin” have seen and approved the manuscript as submitted.

Dr. Javier Alvarez
Professor of Biochemistry
Corresponding author

***Conflict of Interest Statement**

I can certify that there are no conflicts of interest of any kind regarding the manuscript entitled “Monitoring mitochondrial [Ca^{2+}] dynamics with rhod-2, ratiometric pericam and aequorin”.

Dr. Javier Alvarez
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